CONTROL OF GENE EXPRESSION IN PLANTS

This application claims the benefit of U.S. Provisional Application No. 60/242,969, filed October 24, 2000, incorporated herein by reference in its entirety.

CD-R SEQUENCE LISTING

The Sequence Listing associated with the instant disclosure has been submitted as a 434 KB file on CD-R (in duplicate) instead of on paper. Each CD-R is marked in indelible ink to identify the Applicants, Title, File Name (50018A.ST25.txt), Creation Date (October 23, 2001), Computer System (IBM-PC/MS-DOS/MS-Windows), and Docket No. (50018A). The Sequence Listing submitted on CD-R is hereby incorporated by reference into the instant disclosure.

FIELD OF THE INVENTION

The present invention relates to the exogenous control of gene expression in plants. In particular, the present invention relates to chimeric insect hormone receptors and their use for regulation of expression of target polypeptides in plants in the presence of appropriate chemical ligands.

BACKGROUND OF THE INVENTION

The steroid and thyroid hormone superfamily of nuclear receptors is found in mammals and insects and is composed of over 100 known proteins. These receptors fall into at least two functionally distinct categories known as Class I and Class II (Beato, *Cell* 56: 335-344 (1989); Parker, *Sem. Cancer Biol. Ser.* 1: 81-87(1990)). The best studied examples of Class II receptor proteins are Retinoic Acid Receptor (RAR), Vitamin D Receptor (VDR), and Thyroid Hormone Receptor (T_3R) and Retinoic X Receptor (RXR). The receptors bind to the 5' regulatory region of the target gene and, upon binding of a chemical ligand to the receptor, the receptor affects gene expression by interacting with other transcription initiating factors.

In addition to the Class II receptor proteins found in mammals as described above, receptors of similar structure and activity have been identified in insects such as *Drosophila melanogaster* (Koelle *et al., Cell* 67: 59 (1991); Christianson and Kafatos, *Biochem. Biophys. Res. Comm.* 193: 1318 (1993); Henrich *et al., Nucleic Acids Res.* 18: 4143 (1990)). The ecdysone receptor (EcR) binds the steroid hormone 20-hydroxyecdysone (referred to herein as "ecdysone") and, when heterodimerized with the product of the ultraspiracle (USP) gene, transactivates gene expression. USP is most homologous to RXRα, and RXR is capable of forming heterodimers with EcR (Thomas *et al., Nature* 362: 471-475 (1993)).

Class II nuclear receptor polypeptides such as EcR are characterized by the presence of five domains: A/B, C, D, E and F (Evans, R. *Science* 240: 889-895 (1988)), wherein "A/B" refers to the transactivation domain, "C" refers to the DNA binding domain, "D" refers to the hinge/linker domain, "E" refers to the ligand binding domain, and "F" refers to the variable C-terminal domain that is present in some receptor polypeptides.

The "A/B" (transactivation) domain comprises one or more amino acid sequences acting as subdomains that, when combined with the DNA binding domain in a receptor polypeptide, affect the operation of transcription factors during preinitiation and assembly at the TATA box. (See generally, Ptashne, *Nature* 335: 683-689 (1988)). The effect of the transactivation domain is to allow repeated transcription initiation events leading to greater levels of gene expression from a target gene. Different transactivation domains are known to have different degrees of effectiveness in their ability to increase transcription inititiation.

The "C" (DNA binding) domain is a sequence of amino acids having certain functional features that are responsible for binding of the receptor polypeptide to a specific sequence of nucleotides, the response elements, present in the 5' regulatory region of the target gene.

The "D" (hinge/linker) domain is located between the DNA binding domain and the ligand binding domain.

The "E" (ligand binding) domain of the receptor polypeptide provides the means by which the 5' regulatory region of a target gene is activated in response to the presence of a chemical ligand. The ecdysone receptor (EcR) from *Drosophila melanogaster* is one example of a receptor polypeptide where complementary chemical ligands have been identified that bind to the ligand binding domain. The steroid hormone ecdysone triggers coordinate changes in tissue development that results in metamorphosis, and ecdysone has been shown to bind to

EcR (Koelle *et al. Cell* 67: 59-77 (1991)). The plant-produced analog of ecdysone, muristerone, also binds to the ligand binding domain of EcR. Other chemicals, such as the non-steroidal ecdysone agonists RH 5849 (Wing, *Science* 241: 467-469 (1988)) and tebufenozide, the latter known as the insecticide MIMIC®, also will act as a chemical ligand for the ligand binding domain of EcR.

In some cases it is desirable to control the time or extent of expression of a phenotypic trait in plants, plant cells or plant tissue. An ideal situation is the regulation of expression of such a trait at will, triggered by a chemical that can be easily applied to field crops, ornamental shrubs, etc. One such system of regulating gene expression that can be used to achieve this ideal situation is the steroid and thyroid hormone superfamily of nuclear receptors, such as EcR/Ultraspiracle heterodimerized receptors.

U.S. Patent No. 5,880,333, incorporated herein by reference, is drawn to a method of controlling gene expression in plants comprising transforming a plant with at least two receptor expression cassettes and at least one target expression cassette. The first receptor expression cassette comprises a nucleotide sequence for a 5' regulatory region operatively linked to a nucleotide sequence that encodes a first receptor polypeptide operatively linked to a 3' termination region. The second receptor expression cassette comprises a nucleotide sequence for a 5' regulatory region operatively linked to a nucleotide sequence that encodes a second receptor polypeptide operatively linked to a 3' termination region. The first and second receptor polypeptides comprise a first and second ligand binding domain, respectively, which are mutually distinct. The target expression cassette comprises a nucleotide sequence for a 5' regulatory region operatively linked to a nucleotide sequence that encodes a target polypeptide operatively linked to a 3' termination region, wherein the 5' regulatory region of said target expression cassette is activated by said first and second receptor polypeptides in the presence of one or more chemical ligands, whereby expression of said target polypeptide is accomplished. The method is useful for controlling various traits of agronomic importance, such as plant fertility.

However, despite advances such as those described in U.S. Patent No. 5,880,333, there exists a continuing need to develop new and effective systems for inducible gene expression in plants, including the need to develop novel chimeric insect hormone receptors with increased responsiveness to chemical ligands. Especially desirable would be the development

of chimeric class II insect hormone receptors that function in the absence of their normal heterodimerization partners.

SUMMARY OF THE INVENTION

The present invention addresses the aforementioned needs by providing novel chimeric insect hormone receptors and receptor cassettes. The receptor cassettes of the invention are particularly useful for the regulation of expression of target polypeptides in plants in the presence of appropriate chemical ligands. Specifically, each receptor cassette encodes a receptor polypeptide that comprises a DNA binding domain, a hinge region, a ligand binding domain and an activation domain. In a preferred embodiment, the hinge and ligand binding domains are from two different insect ecdysone receptors. In another preferred embodiment, the receptor cassettes are chimeric in that one or more of the DNA binding or activation domains are obtained from a source heterologous with respect to the other domains present in the chimeric receptor cassette.

According to a first aspect, the present invention provides a receptor cassette encoding a chimeric receptor polypeptide comprising: a DNA binding (C) domain; a hinge (D) domain of an ecdysone receptor (EcR) of an insect selected from the group consisting of *Manduca sexta, Agrotis ipsilon, Spodoptera frugiperda, Chironomus tentans,* and *Locusta migratoria*; a ligand binding (E) domain that is heterologous with respect to the hinge (D) domain; and an activation domain. Preferably, the ligand binding (E) domain is a ligand binding (E) domain of an ecdysone receptor of an insect selected from the group consisting of *Manduca sexta, Agrotis ipsilon, Spodoptera frugiperda, Locusta migratoria, Ostrinia nubilalis,* and *Chironomus tentans*. Also, preferably, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain. Preferably, the DNA binding (C) domain is a GAL4 DNA binding domain. According to another preferred embodiment, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain. Preferably, the activation domain is a VP16 activation domain, a maize C1 activation domain, or a maize Dof1 activation domain.

In one embodiment of the receptor cassette described above according to a first aspect of the invention, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Drosophila melanogaster* EcR ligand binding (E) domain. In a

preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Drosophila melanogaster* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-421 of SEQ ID NO:64. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-421 of SEQ ID NO:64. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1263 of SEQ ID NO:63. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1263 of SEQ ID NO:63.

In another embodiment of the receptor cassette described above according to a first aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domainis a *Drosophila melanogaster* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:64. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:64. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:63. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:63.

In another embodiment of the receptor cassette described above according to a first aspect of the invention, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-422 of SEQ ID NO:66. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-422 of SEQ ID NO:66. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid

sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1266 of SEQ ID NO:65. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1266 of SEQ ID NO:65.

In another embodiment of the receptor cassette described above according to a first aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:66. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:66. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:65. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:65.

In a preferred embodiment of the receptor cassette described above according to a first aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-511 of SEQ ID NO:119. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-511 of SEQ ID NO:119. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1533 of SEQ ID NO:118. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1533 of SEQ ID NO:118.

In another preferred embodiment of the receptor cassette described above according to a first aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:119. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:119. In a particularly preferred embodiment, the

receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:118. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:118.

In another embodiment of the receptor cassette described above according to a first aspect of the invention, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-419 of SEQ ID NO:68. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-419 of SEQ ID NO:68. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1257 of SEQ ID NO:67. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1257 of SEQ ID NO:67.

In a preferred embodiment of the receptor cassette described above according to a first aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-508 of SEQ ID NO:121. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-508 of SEQ ID NO:121. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1524 of SEQ ID NO:120. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1524 of SEQ ID NO:120. In another preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. According to this embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least

90% identical to SEQ ID NO:121. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:121. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:120. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:120.

In another embodiment of the receptor cassette described above according to a first aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:68. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:68. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:67. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:67.

In another embodiment of the receptor cassette described above according to a first aspect of the invention, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Spodoptera frugiperda* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Spodoptera frugiperda* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-419 of SEQ ID NO:70. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-419 of SEQ ID NO:70. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1257 of SEQ ID NO:69. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1257 of SEQ ID NO:69.

In another embodiment of the receptor cassette described above according to a first aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand

binding (E) domain is a *Spodoptera frugiperda* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In a preferred embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:70. In a more preferred embodiment, the chimeric receptor polypeptide comprises SEQ ID NO:70. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:69. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:69.

In a preferred embodiment of the receptor cassette described above according to a first aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Spodoptera frugiperda* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-508 of SEQ ID NO:123. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-508 of SEQ ID NO:123. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1524 of SEQ ID NO:122. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1524 of SEQ ID NO:122.

In a preferred embodiment of the receptor cassette described above according to a first aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is a *Spodoptera frugiperda* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In a preferred embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:123. In a more preferred embodiment, the chimeric receptor polypeptide comprises SEQ ID NO:123. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:122. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:122.

In another embodiment of the receptor cassette described above according to a first aspect of the invention, the hinge (D) domain is a *Locusta migratoria* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. In a

preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is a *Locusta migratoria* EcR DNA binding (C) domain, the hinge (D) domain is a *Locusta migratoria* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. In a preferred embodiment, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-407 of SEQ ID NO:84. In a more preferred embodiment, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-407 of SEQ ID NO:84. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1221 of SEQ ID NO:83. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1221 of SEQ ID NO:83.

In another embodiment of the receptor cassette described above according to a first aspect of the invention, the DNA binding (C) domain is a *Locusta migratoria* EcR DNA binding (C) domain, the hinge (D) domain is a *Locusta migratoria* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In a preferred embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:84. In a more preferred embodiment, the chimeric receptor polypeptide comprises SEQ ID NO:84. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:83. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:83.

In another embodiment of the receptor cassette described above according to a first aspect of the invention, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Locusta migratoria* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Locusta migratoria* EcR ligand binding (E) domain. In a preferred embodiment, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-416 of SEQ ID

NO:86. In a more preferred embodiment, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-416 of SEQ ID NO:86. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1248 of SEQ ID NO:85. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1248 of SEQ ID NO:85.

In another embodiment of the receptor cassette described above according to a first aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is a *Locusta migratoria* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:86. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:86. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:85. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:85.

In another embodiment of the receptor cassette described above according to a first aspect of the invention, the hinge (D) domain is a *Chironomus tentans* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is a *Chironomus tentans* EcR DNA binding (C) domain, the hinge (D) domain is a *Chironomus tentans* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-441 of SEQ ID NO:90. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-441 of SEQ ID NO:90. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1323 of SEQ ID NO:89. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1323 of SEQ ID NO:89.

In another embodiment of the receptor cassette described above according to a first aspect of the invention, the DNA binding (C) domain is a *Chironomus tentans* EcR DNA binding (C) domain, the hinge (D) domain is a *Chironomus tentans* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:90. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:90. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:89. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:89.

In another embodiment of the receptor cassette described above according to a first aspect of the invention, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Chironomus tentans* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Chironomus tentans* EcR hinge (D) domain, and the ligand binding (E) domain is a *Chironomus tentans* EcR ligand binding (E) domain. In a more preferred embodiment, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-420 of SEQ ID NO:92. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-420 of SEQ ID NO:92. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1260 of SEQ ID NO:91. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1260 of SEQ ID NO:91.

In another embodiment of the receptor cassette described above according to a first aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Chironomus tentans* EcR hinge (D) domain, the ligand binding (E) domain is a *Chironomus tentans* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In a preferred embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:92. In another preferred embodiment, the chimeric receptor polypeptide comprises SEQ

ID NO:92. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:91. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:91.

According to a second aspect, the present invention provides a receptor cassette encoding a chimeric receptor polypeptide comprising: a DNA binding (C) domain; a hinge (D) domain; a ligand binding (E) domain of an ecdysone receptor (EcR) of an insect selected from the group consisting of *Manduca sexta, Agrotis ipsilon, Spodoptera frugiperda, Chironomus tentans,* and *Locusta migratoria,* wherein the ligand binding (E) domain is heterologous with respect to the hinge (D) domain; and an activation domain. In a preferred embodiment, the hinge (D) domain is a hinge (D) domain of an ecdysone receptor of an insect selected from the group consisting of *Manduca sexta, Agrotis ipsilon, Spodoptera frugiperda, Locusta migratoria, Ostrinia nubilalis,* and *Chironomus tentans.* In another preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. In another preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain.

In one embodiment of the receptor cassette described above according to a second aspect of the invention, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, and the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is an *Ostrinia nubilalis* EcR DNA binding (C) domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, and the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E). Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-427 of SEQ ID NO:78. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-427 of SEQ ID NO:78. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1281 of SEQ ID NO:77. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1281 of SEQ ID NO:77.

In another embodiment of the receptor cassette described above according to a second aspect of the invention, the DNA binding (C) domain is an *Ostrinia nubilalis* EcR DNA binding (C) domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:78. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:78. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:77. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:77.

In another embodiment of the receptor cassette described above according to a second aspect of the invention, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is an *Ostrinia nubilalis* EcR DNA binding (C) domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-430 of SEQ ID NO:80. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-430 of SEQ ID NO:80. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1290 of SEQ ID NO:79. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1290 of SEQ ID NO:79.

In another embodiment of the receptor cassette described above according to a second aspect of the invention, the DNA binding (C) domain is an *Ostrinia nubilalis* EcR DNA binding (C) domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:80. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:80. In an especially preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the

complement hybridizes under stringent conditions to SEQ ID NO:79. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:79.

In a preferred embodiment of the receptor cassette described above according to a second aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-519 of SEQ ID NO:127. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-519 of SEQ ID NO:127. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1557 of SEQ ID NO:126. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1557 of SEQ ID NO:126.

In another preferred embodiment of the receptor cassette described above according to a second aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:127. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:127. In an especially preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:126. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:126.

In another embodiment of the receptor cassette described above according to a second aspect of the invention, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is a *Drosophila melanogaster* EcR DNA binding (C) domain, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor

polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-436 of SEQ ID NO:72. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-436 of SEQ ID NO:72. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1308 of SEQ ID NO:71. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1308 of SEQ ID NO:71.

In another embodiment of the receptor cassette described above according to a second aspect of the invention, the DNA binding (C) domain is a *Drosophila melanogaster* EcR DNA binding (C) domain, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In a preferred embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:72. In a more preferred embodiment, the chimeric receptor polypeptide comprises SEQ ID NO:72. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:71. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:71.

In another embodiment of the receptor cassette described above according to a second aspect of the invention, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, and the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is a *Drosophila melanogaster* EcR DNA binding (C) domain, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, and the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-433 of SEQ ID NO:74. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-433 of SEQ ID NO:74. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1299 of SEQ ID NO:73. In

an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1299 of SEQ ID NO:73.

In another embodiment of the receptor cassette described above according to a second aspect of the invention, the DNA binding (C) domain is a *Drosophila melanogaster* EcR DNA binding (C) domain, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In a preferred embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:74. In a more preferred embodiment, the chimeric receptor polypeptide comprises SEQ ID NO:74. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:73. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:73.

According to a third aspect, the present invention provides a receptor cassette encoding a chimeric receptor polypeptide comprising: a GAL4 DNA binding domain or a DNA binding (C) domain of an ecdysone receptor (EcR) of an insect selected from the group consisting of *Ostrinia nubilalis, Locusta migratoria, Chironomus tentans, Manduca sexta,* and *Drosophila melanogaster*; a hinge (D) domain of an ecdysone receptor of an insect selected from the group consisting of *Ostrinia nubilalis, Locusta migratoria, Chironomus tentans, Manduca sexta,* and *Drosophila melanogaster*; a ligand binding (E) domain of an ecdysone receptor of an insect selected from the group consisting of *Ostrinia nubilalis, Locusta migratoria, Chironomus tentans, Manduca sexta,* and *Drosophila melanogaster*; and a heterologous activation domain; wherein the chimeric receptor polypeptide does not include an ecdysone receptor A/B N-terminal domain. In a particularly preferred embodiment, the receptor cassette encodes a chimeric receptor polypeptide that consists essentially of: a GAL4 DNA binding domain or a DNA binding (C) domain of an ecdysone receptor (EcR) of an insect selected from the group consisting of *Ostrinia nubilalis, Locusta migratoria, Chironomus tentans, Manduca sexta,* and *Drosophila melanogaster*; a hinge (D) domain of an ecdysone receptor of an insect selected from the group consisting of *Ostrinia nubilalis, Locusta migratoria, Chironomus tentans, Manduca sexta,* and *Drosophila melanogaster*; a ligand binding (E) domain of an ecdysone receptor of an insect selected from the group consisting of *Ostrinia*

nubilalis, Locusta migratoria, Chironomus tentans, Manduca sexta, and *Drosophila melanogaster*; and a heterologous activation domain that is not an ecdysone receptor A/B N-terminal domain. In one preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. Also, in another preferred embodiment, the activation domain is a VP16 activation domain, a maize C1 activation domain, or a maize Dof1 activation domain.

In one embodiment of the receptor cassette described above according to a third aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. 23. In one configuration, the VP16 activation domain is located at the N-terminus of the chimeric receptor polypeptide. In another configuration, the VP16 activation domain is located internally in the chimeric receptor polypeptide between the GAL4 DNA binding domain and the *Manduca sexta* EcR hinge (D) domain. In yet another configuration, the VP16 activation domain is located at the C-terminus of the chimeric receptor polypeptide. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:105. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:105. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 2007-3668 of SEQ ID NO:104. In an especially preferred embodiment, the receptor cassette comprises nucleotides 2007-3668 of SEQ ID NO:104.

In another embodiment of the receptor cassette described above according to a third aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, the ligand binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:125. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:125. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:124. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:124.

In another embodiment of the receptor cassette described above according to a third aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a maize C1 activation domain. According to this embodiment, the chimeric receptor polypeptide preferably comprises an amino acid sequence at least 90% identical to SEQ ID NO:135. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:135. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:134. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:134.

In another embodiment of the receptor cassette described above according to a third aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a maize Dof1 activation domain. According to this embodiment, the chimeric receptor polypeptide preferably comprises an amino acid sequence at least 90% identical to SEQ ID NO:137. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:137. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:136. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:136.

In another embodiment of the receptor cassette described above according to a third aspect of the invention, the activation domain is an N-terminal VP16 activation domain, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. According to this embodiment, the chimeric receptor polypeptide preferably comprises an amino acid sequence at least 90% identical to SEQ ID NO:143. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:143. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:142. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:142.

In another embodiment of the receptor cassette described above according to a third aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the activation domain is an internally configured VP16 activation domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. According to this embodiment, the chimeric receptor polypeptide preferably comprises an amino acid sequence at least 90% identical to SEQ ID NO:148. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:148. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:147. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:147.

In another embodiment of the receptor cassette described above according to a third aspect of the invention, the DNA binding (C) domain is an *Ostrinia nubilalis* EcR DNA binding (C) domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, and the ligand binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E) domain. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-424 of SEQ ID NO:76. In a more preferred embodiment, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-424 of SEQ ID NO:76. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1272 of SEQ ID NO:75. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1272 of SEQ ID NO:75.

In another embodiment of the receptor cassette described above according to a third aspect of the invention, the DNA binding (C) domain is an *Ostrinia nubilalis* EcR DNA binding (C) domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, the ligand binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:76. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:76. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:75. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:75.

In another embodiment of the receptor cassette described above according to a third aspect of the invention, the DNA binding (C) domain is a *Locusta migratoria* EcR DNA binding (C) domain, the hinge (D) domain is a *Locusta migratoria* EcR hinge (D) domain, and the ligand binding (E) domain is a *Locusta migratoria* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-398 of SEQ ID NO:82. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-398 of SEQ ID NO:82. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1194 of SEQ ID NO:81. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1194 of SEQ ID NO:81.

In another embodiment of the receptor cassette described above according to a third aspect of the invention, the DNA binding (C) domain is a *Locusta migratoria* EcR DNA binding (C) domain, the hinge (D) domain is a *Locusta migratoria* EcR hinge (D) domain, the ligand binding (E) domain is a *Locusta migratoria* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:82. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:82. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:81. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:81.

In another embodiment of the receptor cassette described above according to a third aspect of the invention, the DNA binding (C) domain is a *Chironomus tentans* EcR DNA binding (C) domain, the hinge (D) domain is a *Chironomus tentans* EcR hinge (D) domain, and the ligand binding (E) domain is a *Chironomus tentans* EcR ligand binding (E) domain. In a preferred embodiment, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-436 of SEQ ID NO:88. In a more preferred embodiment, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-436 of SEQ ID NO:88. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1308 of SEQ ID NO:87. In

an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1308 of SEQ ID NO:87.

In another embodiment of the receptor cassette described above according to a third aspect of the invention, the DNA binding (C) domain is a *Chironomus tentans* EcR DNA binding (C) domain, the hinge (D) domain is a *Chironomus tentans* EcR hinge (D) domain, the ligand binding (E) domain is a *Chironomus tentans* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:88. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:88. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:87. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:87.

In another embodiment of the receptor cassette described above according to a third aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. In a preferred embodiment, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-425 of SEQ ID NO:94. In a more preferred embodiment, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-425 of SEQ ID NO:94. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1275 of SEQ ID NO:93. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1275 of SEQ ID NO:93.

In another embodiment of the receptor cassette described above according to a third aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:94. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:94. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the

complement hybridizes under stringent conditions to SEQ ID NO:93. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:93.

In another embodiment of the receptor cassette described above according to a third aspect of the invention, the DNA binding (C) domain is a *Drosophila melanogaster* EcR DNA binding (C) domain, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, and the ligand binding (E) domain is an *Drosophila melanogaster* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-432 of SEQ ID NO:96. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-432 of SEQ ID NO:96. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to nucleotides 1-1296 of SEQ ID NO:95. In an especially preferred embodiment, the receptor cassette comprises nucleotides 1-1296 of SEQ ID NO:95

In another embodiment of the receptor cassette described above according to a third aspect of the invention, the DNA binding (C) domain is a *Drosophila melanogaster* EcR DNA binding (C) domain, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, the ligand binding (E) domain is an *Drosophila melanogaster* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:96. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:96. In a particularly preferred embodiment, the receptor cassette comprises a nucleic acid sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:95. In an especially preferred embodiment, the receptor cassette comprises SEQ ID NO:95

According to a fourth aspect, the present invention provides a receptor cassette encoding a chimeric receptor polypeptide comprising: at least one DNA binding (C) domain; a hinge (D) domain of an insect ecdysone receptor (EcR); a ligand binding (E) domain of an insect ecdysone receptor, wherein the ligand binding (E) domain is heterologous with respect to the hinge (D) domain; and a heterologous activation domain; wherein the chimeric receptor polypeptide does not include an ecdysone receptor A/B N-terminal domain. In a particularly preferred embodiment, the receptor cassette encodes a chimeric receptor polypeptide that consists essentially of: at least one DNA binding (C) domain; a hinge (D) domain of an insect

ecdysone receptor; a ligand binding (E) domain of an insect ecdysone receptor, wherein the ligand binding (E) domain is heterologous with respect to the hinge (D) domain; and a heterologous activation domain that is not an ecdysone receptor A/B N-terminal domain. Preferably, the DNA binding (C) domain is a GAL4 DNA binding domain. Also, preferably, the activation domain is a VP16 activation domain, a maize C1 activation domain, or a maize Dof1 activation domain.

According to a fifth aspect, the present invention provides a receptor cassette encoding a chimeric receptor polypeptide comprising: a DNA binding (C) domain; a hinge (D) domain of an insect ecdysone receptor (EcR); a ligand binding (E) domain of an ecdysone receptor of a lepidopteran insect other than *Bombyx mori*, wherein the ligand binding (E) domain is heterologous with respect to the hinge (D) domain; and an activation domain. Preferably, the DNA binding (C) domain is a GAL4 DNA binding domain. Also, preferably, the activation domain is a VP16 activation domain, a maize C1 activation domain, or a maize Dof1 activation domain. Still further, preferably, the hinge (D) domain is the hinge (D) domain of a lepidopteran insect ecdysone receptor.

According to a sixth aspect, the present invention provides an isolated nucleic acid molecule comprising a nucleotide sequence that encodes an ecdysone receptor of *Spodoptera frugiperda* or *Agrotis ipsilon*. Preferably, the ecdysone receptor comprises an amino acid sequence at least 90% identical to SEQ ID NO:8 or SEQ ID NO:10. More preferably, the ecdysone receptor comprises SEQ ID NO:8 or SEQ ID NO:10. More preferably, the isolated nucleic acid molecule comprises a nucleotide sequence of which the complement hybridizes under stringent conditions to SEQ ID NO:7 or SEQ ID NO:9. Even more preferably, the isolated nucleic acid molecule comprises SEQ ID NO:7 or SEQ ID NO:9.

Additional aspects of the present invention involve a receptor expression cassette comprising a heterologous promoter sequence operatively linked to any of the above-described receptor cassettes of the invention; a recombinant vector comprising a receptor expression cassette according to the invention; and a transgenic host cell comprising a receptor expression cassette according to the invention. Preferably, the transgenic host cell is a plant cell. Yet additional aspects of the present invention involve a transgenic plant comprising such a transgenic plant cell, and seed from such a transgenic plant. Transgenic plants according to the present invention may be monocots or dicots and include, but are not

limited to, maize, wheat, barley, rye, sweet potato, bean, pea, chicory, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, pepper, celery, squash, pumpkin, hemp, zucchini, apple, pear, quince, melon (e.g., watermelon), plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tomato, sorghum, sugarcane, sugarbeet, sunflower, rapeseed, clover, tobacco, carrot, cotton, alfalfa, rice, potato, eggplant, cucumber, *Arabidopsis*, and woody plants such as coniferous and deciduous trees.

A twelfth aspect of the present invention concerns a method of controlling gene expression in a plant, comprising: transforming the plant with a receptor expression cassette comprising a 5' regulatory region capable of promoting expression in a plant cell operatively linked to a receptor cassette of the invention as described above, and a 3' terminating region; and a target expression cassette comprising a 5' regulatory region operatively linked to a target nucleotide sequence, wherein the 5' regulatory region comprises one or more response elements complementary to the DNA binding (C) domain of the chimeric receptor polypeptide; expressing the chimeric receptor polypeptide in the plant; contacting the plant with a chemical ligand that is complementary to the ligand binding (E) domain of the chimeric receptor polypeptide, whereby the chimeric receptor polypeptide in the presence of the chemical ligand activates expression of the target nucleotide sequence. Preferably, the ligand binding (E) domain of the chimeric receptor polypeptide is a *Manduca sexta* EcR ligand binding (E) domain. Also, preferably, the chemical ligand is tebufenozide or methoxytebufenozide.

In an especially preferred embodiment, the present invention provides a method of controlling gene expression in a plant, comprising: a) transforming the plant with (i) a receptor expression cassette comprising a 5' regulatory region capable of promoting expression in a plant cell operatively linked to a receptor cassette encoding a chimeric receptor polypeptide comprising a DNA binding (C) domain, a hinge (D) domain of a *Manduca sexta* ecdysone receptor, a ligand binding (E) domain of a *Manduca sexta* ecdysone receptor, and an activation domain, and a 3' terminating region; and (ii) a target expression cassette comprising a 5' regulatory region operatively linked to a target nucleotide sequence, wherein the 5' regulatory region comprises one or more response elements complementary to the DNA binding (C) domain of the chimeric receptor polypeptide; b) expressing the chimeric

receptor polypeptide in the plant; c) contacting the plant with a chemical ligand that is complementary to the ligand binding (E) domain of the chimeric receptor polypeptide, whereby the chimeric receptor polypeptide in the presence of the chemical ligand activates expression of the target nucleotide sequence. Preferably, the DNA binding (C) domain is a GAL4 DNA binding domain. Also, preferably, the activation domain of the chimeric receptor polypeptide is a VP16 activation domain, a maize C1 activation domain, or a maize Dof1 activation domain. Still further, preferably, the chemical ligand is tebufenozide or methoxytebufenozide.

According to a thirteenth aspect, the present invention provides a chimeric receptor polypeptide comprising: a DNA binding (C) domain; a hinge (D) domain of an ecdysone receptor (EcR) of an insect selected from the group consisting of *Manduca sexta, Agrotis ipsilon, Spodoptera frugiperda, Chironomus tentans,* and *Locusta migratoria*; a ligand binding (E) domain that is heterologous with respect to the hinge (D) domain; and an activation domain. In a preferred embodiment, the ligand binding (E) domain is a ligand binding (E) domain of an ecdysone receptor of an insect selected from the group consisting of *Manduca sexta, Agrotis ipsilon, Spodoptera frugiperda, Locusta migratoria, Ostrinia nubilalis,* and *Chironomus tentans*. In another preferred embodiment, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain. Preferably, the activation domain is a VP16 activation domain, a maize C1 activation domain, or a maize Dof1 activation domain.

In one embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Drosophila melanogaster* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Drosophila melanogaster* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-421 of

SEQ ID NO:64. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-421 of SEQ ID NO:64.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is a *Drosophila melanogaster* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In a preferred embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:64. In a more preferred embodiment, the chimeric receptor polypeptide comprises SEQ ID NO:64.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-422 of SEQ ID NO:66. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-422 of SEQ ID NO:66.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:66. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:66.

In a preferred embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain.

Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-511 of SEQ ID NO:119. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-511 of SEQ ID NO:119.

In another preferred embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the DNA binding domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:119. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:119.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-419 of SEQ ID NO:68. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-419 of SEQ ID NO:68.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:68. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:68.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand

binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-508 of SEQ ID NO:121. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-508 of SEQ ID NO:121.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. According to this embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:121. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:121.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Spodoptera frugiperda* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Spodoptera frugiperda* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-419 of SEQ ID NO:70. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-419 of SEQ ID NO:70.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is a *Spodoptera frugiperda* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In a preferred embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:70. In a more preferred embodiment, the chimeric receptor polypeptide comprises SEQ ID NO:70.

In a preferred embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Spodoptera frugiperda* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-508 of SEQ ID NO:123. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-508 of SEQ ID NO:123.

In another preferred embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is a *Spodoptera frugiperda* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In a preferred embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:123. In a more preferred embodiment, the chimeric receptor polypeptide comprises SEQ ID NO:123.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the hinge (D) domain is a *Locusta migratoria* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is a *Locusta migratoria* EcR DNA binding (C) domain, the hinge (D) domain is a *Locusta migratoria* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-407 of SEQ ID NO:84.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the DNA binding (C) domain is a *Locusta migratoria* EcR DNA binding (C) domain, the hinge (D) domain is a *Locusta migratoria* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In a preferred embodiment, the

chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:84. In another preferred embodiment, the chimeric receptor polypeptide comprises SEQ ID NO:84.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Locusta migratoria* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Locusta migratoria* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-416 of SEQ ID NO:86. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-416 of SEQ ID NO:86.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is a *Locusta migratoria* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:86. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:86.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the hinge (D) domain is a *Chironomus tentans* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is a *Chironomus tentans* EcR DNA binding (C) domain, the hinge (D) domain is a *Chironomus tentans* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-441 of

SEQ ID NO:90. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-441 of SEQ ID NO:90.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the DNA binding (C) domain is a *Chironomus tentans* EcR DNA binding (C) domain, the hinge (D) domain is a *Chironomus tentans* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In a preferred embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:90. In a more preferred embodiment, the chimeric receptor polypeptide comprises SEQ ID NO:90.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Chironomus tentans* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Chironomus tentans* EcR hinge (D) domain, and the ligand binding (E) domain is a *Chironomus tentans* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-420 of SEQ ID NO:92. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-420 of SEQ ID NO:92.

In another embodiment of the chimeric receptor polypeptide described above according to a thirteenth aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Chironomus tentans* EcR hinge (D) domain, the ligand binding (E) domain is a *Chironomus tentans* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In a preferred embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:92. In a more preferred embodiment, the chimeric receptor polypeptide comprises SEQ ID NO:92.

According to a fourteenth aspect, the present invention provides a chimeric receptor polypeptide comprising: a DNA binding (C) domain; a hinge (D) domain; a ligand binding (E)

In another embodiment of the chimeric receptor polypeptide described above according to a fourteenth aspect of the invention, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is an *Ostrinia nubilalis* EcR DNA binding (C) domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-430 of SEQ ID NO:80. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-430 of SEQ ID NO:80.

In another embodiment of the chimeric receptor polypeptide described above according to a fourteenth aspect of the invention, the DNA binding (C) domain is an *Ostrinia nubilalis* EcR DNA binding (C) domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:80. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:80.

In a preferred embodiment of the chimeric receptor polypeptide described above according to a fourteenth aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-519 of SEQ ID NO:127. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-519 of SEQ ID NO:127.

In another preferred embodiment of the chimeric receptor polypeptide described above according to a fourteenth aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide

domain of an ecdysone receptor (EcR) of an insect selected from the group consisting of *Manduca sexta, Agrotis ipsilon, Spodoptera frugiperda, Chironomus tentans,* and *Locusta migratoria*, wherein the ligand binding (E) domain is heterologous with respect to the hinge (D) domain; and an activation domain.

In one embodiment of the chimeric receptor polypeptide described above according to a fourteenth aspect of the invention, the hinge (D) domain is a hinge (D) domain of an ecdysone receptor of an insect selected from the group consisting of *Manduca sexta, Agrotis ipsilon, Spodoptera frugiperda, Locusta migratoria, Ostrinia nubilalis,* and *Chironomus tentans*. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the ligand binding (E) domain is a *Manduca sexta* ligand binding (E) domain. In another preferred embodiment, the activation domain is a VP16 activation domain, a maize C1 activation domain, or a maize Dof1 activation domain.

In another embodiment of the chimeric receptor polypeptide described above according to a fourteenth aspect of the invention, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, and the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is an *Ostrinia nubilalis* EcR DNA binding (C) domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, and the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-427 of SEQ ID NO:78. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-427 of SEQ ID NO:78.

In another embodiment of the chimeric receptor polypeptide described above according to a fourteenth aspect of the invention, the DNA binding (C) domain is an *Ostrinia nubilalis* EcR DNA binding (C) domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In a preferred embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:78. In a more preferred embodiment, the chimeric receptor polypeptide comprises SEQ ID NO:78.

comprises an amino acid sequence at least 90% identical to SEQ ID NO:127. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:127.

In another embodiment of the chimeric receptor polypeptide described above according to a fourteenth aspect of the invention, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is a *Drosophila melanogaster* EcR DNA binding (C) domain, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-436 of SEQ ID NO:72. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-436 of SEQ ID NO:72.

In another embodiment of the chimeric receptor polypeptide described above according to a fourteenth aspect of the invention, the DNA binding (C) domain is a *Drosophila melanogaster* EcR DNA binding (C) domain, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In a preferred embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:72. In a more preferred embodiment, the chimeric receptor polypeptide comprises SEQ ID NO:72.

In another embodiment of the chimeric receptor polypeptide described above according to a fourteenth aspect of the invention, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, and the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain. In a preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. In another preferred embodiment, the DNA binding (C) domain is a *Drosophila melanogaster* EcR DNA binding (C) domain, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, and the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to

amino acids 1-433 of SEQ ID NO:74. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-433 of SEQ ID NO:74.

In another embodiment of the chimeric receptor polypeptide described above according to a fourteenth aspect of the invention, the DNA binding (C) domain is a *Drosophila melanogaster* EcR DNA binding (C) domain, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, the ligand binding (E) domain is an *Agrotis ipsilon* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:74. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:74.

According to an fifteenth aspect, the present invention provides a chimeric receptor polypeptide comprising: a GAL4 DNA binding domain or a DNA binding (C) domain of an ecdysone receptor (EcR) of an insect selected from the group consisting of *Ostrinia nubilalis, Locusta migratoria, Chironomus tentans, Manduca sexta,* and *Drosophila melanogaster*; a hinge (D) domain of an ecdysone receptor of an insect selected from the group consisting of *Ostrinia nubilalis, Locusta migratoria, Chironomus tentans, Manduca sexta,* and *Drosophila melanogaster*; a ligand binding (E) domain of an ecdysone receptor of an insect selected from the group consisting of *Ostrinia nubilalis, Locusta migratoria, Chironomus tentans, Manduca sexta,* and *Drosophila melanogaster*; and a heterologous activation domain; wherein the chimeric receptor polypeptide does not include an ecdysone receptor A/B N-terminal domain. In a particularly preferred embodiment, the chimeric receptor polypeptide consists essentially of: a GAL4 DNA binding domain or a DNA binding (C) domain of an ecdysone receptor (EcR) of an insect selected from the group consisting of *Ostrinia nubilalis, Locusta migratoria, Chironomus tentans, Manduca sexta,* and *Drosophila melanogaster*; a hinge (D) domain of an ecdysone receptor of an insect selected from the group consisting of *Ostrinia nubilalis, Locusta migratoria, Chironomus tentans, Manduca sexta,* and *Drosophila melanogaster*; a ligand binding (E) domain of an ecdysone receptor of an insect selected from the group consisting of *Ostrinia nubilalis, Locusta migratoria, Chironomus tentans, Manduca sexta,* and *Drosophila melanogaster*; and a heterologous activation domain that is not an ecdysone receptor A/B N-terminal domain. In one preferred embodiment, the DNA binding (C) domain is a GAL4 DNA binding domain. Also, in another preferred embodiment, the

activation domain is a VP16 activation domain, a maize C1 activation domain, or a maize Dof1 activation domain.

In one embodiment of the chimeric receptor polypeptide described above according to a fifteenth aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In one configuration, the VP16 activation domain is located at the N-terminus of the chimeric receptor polypeptide. In another configuration, the VP16 activation domain is located internally in the chimeric receptor polypeptide between the GAL4 DNA binding domain and the *Manduca sexta* EcR hinge (D) domain. In yet another configuration, the VP16 activation domain is located at the C-terminus of the chimeric receptor polypeptide. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:105. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:105.

In another embodiment of the chimeric receptor polypeptide described above according to a fifteenth aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, the ligand binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:125. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:125.

In another embodiment of the chimeric receptor polypeptide described above according to a fifteenth aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a maize C1 activation domain. According to this embodiment, the chimeric receptor polypeptide preferably comprises an amino acid sequence at least 90% identical to SEQ ID NO:135. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:135.

In another embodiment of the chimeric receptor polypeptide described above according to a fifteenth aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding

(E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a maize Dof1 activation domain. According to this embodiment, the chimeric receptor polypeptide preferably comprises an amino acid sequence at least 90% identical to SEQ ID NO:137. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:137.

In another embodiment of the chimeric receptor polypeptide described above according to a fifteenth aspect of the invention, the activation domain is an N-terminal VP16 activation domain, the DNA binding (C) domain is a GAL4 DNA binding domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. According to this embodiment, the chimeric receptor polypeptide preferably comprises an amino acid sequence at least 90% identical to SEQ ID NO:143. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:143.

In another embodiment of the chimeric receptor polypeptide described above according to a fifteenth aspect of the invention, the DNA binding (C) domain is a GAL4 DNA binding domain, the activation domain is an internally configured VP16 activation domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. According to this embodiment, the chimeric receptor polypeptide preferably comprises an amino acid sequence at least 90% identical to SEQ ID NO:148. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:148.

In one embodiment of the chimeric receptor polypeptide described above according to an fifteenth aspect of the invention, the DNA binding (C) domain is an *Ostrinia nubilalis* EcR DNA binding (C) domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, and the ligand binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E) domain. In a preferred embodiment, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-424 of SEQ ID NO:76. In a more preferred embodiment, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-424 of SEQ ID NO:76.

In another embodiment of the chimeric receptor polypeptide described above according to an fifteenth aspect of the invention, the DNA binding (C) domain is an *Ostrinia nubilalis* EcR DNA binding (C) domain, the hinge (D) domain is an *Ostrinia nubilalis* EcR hinge (D) domain, the ligand binding (E) domain is an *Ostrinia nubilalis* EcR ligand binding (E)

domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:76. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:76.

In another embodiment of the chimeric receptor polypeptide described above according to an fifteenth aspect of the invention, the DNA binding (C) domain is a *Locusta migratoria* EcR DNA binding (C) domain, the hinge (D) domain is a *Locusta migratoria* EcR hinge (D) domain, and the ligand binding (E) domain is a *Locusta migratoria* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-398 of SEQ ID NO:82. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-398 of SEQ ID NO:82.

In another embodiment of the chimeric receptor polypeptide described above according to an fifteenth aspect of the invention, the DNA binding (C) domain is a *Locusta migratoria* EcR DNA binding (C) domain, the hinge (D) domain is a *Locusta migratoria* EcR hinge (D) domain, the ligand binding (E) domain is a *Locusta migratoria* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. In a preferred embodiment, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:82. In a more preferred embodiment, the chimeric receptor polypeptide comprises SEQ ID NO:82.

In another embodiment of the chimeric receptor polypeptide described above according to an fifteenth aspect of the invention, the DNA binding (C) domain is a *Chironomus tentans* EcR DNA binding (C) domain, the hinge (D) domain is a *Chironomus tentans* EcR hinge (D) domain, and the ligand binding (E) domain is a *Chironomus tentans* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-436 of SEQ ID NO:88. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-436 of SEQ ID NO:88.

In another embodiment of the chimeric receptor polypeptide described above according to an fifteenth aspect of the invention, the DNA binding (C) domain is a *Chironomus tentans* EcR DNA binding (C) domain, the hinge (D) domain is a *Chironomus tentans* EcR hinge (D) domain, the ligand binding (E) domain is a *Chironomus tentans* EcR ligand binding (E)

domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:88. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:88.

In another embodiment of the chimeric receptor polypeptide described above according to an fifteenth aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, and the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-425 of SEQ ID NO:94. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-425 of SEQ ID NO:94.

In another embodiment of the chimeric receptor polypeptide described above according to an fifteenth aspect of the invention, the DNA binding (C) domain is a *Manduca sexta* EcR DNA binding (C) domain, the hinge (D) domain is a *Manduca sexta* EcR hinge (D) domain, the ligand binding (E) domain is a *Manduca sexta* EcR ligand binding (E) domain, and the activation domain is a VP16 activation domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:94. More preferably, the chimeric receptor polypeptide comprises SEQ ID NO:94.

In another embodiment of the chimeric receptor polypeptide described above according to an fifteenth aspect of the invention, the DNA binding (C) domain is a *Drosophila melanogaster* EcR DNA binding (C) domain, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, and the ligand binding (E) domain is an *Drosophila melanogaster* EcR ligand binding (E) domain. Preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise an amino acid sequence at least 90% identical to amino acids 1-432 of SEQ ID NO:96. More preferably, the C, D, and E domains of the chimeric receptor polypeptide comprise amino acids 1-432 of SEQ ID NO:96.

In another embodiment of the chimeric receptor polypeptide described above according to an fifteenth aspect of the invention, the DNA binding (C) domain is a *Drosophila melanogaster* EcR DNA binding (C) domain, the hinge (D) domain is a *Drosophila melanogaster* EcR hinge (D) domain, the ligand binding (E) domain is an *Drosophila melanogaster* EcR ligand binding (E) domain, and the activation domain is a VP16 activation

domain. Preferably, the chimeric receptor polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:96. Preferably, the chimeric receptor polypeptide comprises SEQ ID NO:96.

According to a sixteenth aspect, the present invention provides a chimeric receptor polypeptide comprising: at least one DNA binding (C) domain; a hinge (D) domain of an insect ecdysone receptor (EcR); a ligand binding (E) domain of an insect ecdysone receptor, wherein the ligand binding (E) domain is heterologous with respect to the hinge (D) domain; and a heterologous activation domain; wherein the chimeric receptor polypeptide does not include an ecdysone receptor A/B N-terminal domain. In a particulary preferred embodiment, the chimeric receptor polypeptide consists essentially of: at least one DNA binding (C) domain; a hinge (D) domain of an insect ecdysone receptor; a ligand binding (E) domain of an insect ecdysone receptor, wherein the ligand binding (E) domain is heterologous with respect to the hinge (D) domain; and a heterologous activation domain that is not an ecdysone receptor A/B N-terminal domain. Preferably, the DNA binding (C) domain is a GAL4 DNA binding domain. Also, preferably, the activation domain is a VP16 activation domain, a maize C1 activation domain, or a maize Dof1 activation domain.

According to a seventeenth aspect, the present invention provides a chimeric receptor polypeptide comprising: a DNA binding (C) domain; a hinge (D) domain of an insect ecdysone receptor (EcR); a ligand binding (E) domain of an ecdysone receptor of a lepidopteran insect other than *Bombyx mori*, wherein the ligand binding (E) domain is heterologous with respect to the hinge (D) domain; and an activation domain. Preferably, the DNA binding (C) domain is a GAL4 DNA binding domain. Preferably, the activation domain is a VP16 activation domain, a maize C1 activation domain, or a maize Dof1 activation domain. In a preferred embodiment, the hinge (D) domain is the hinge (D) domain of a lepidopteran insect ecdysone receptor.

According to a eighteenth aspect, the present invention provides an isolated ecdysone receptor of *Spodoptera frugiperda* or *Agrotis ipsilon*. Preferably, such a receptor comprises an amino acid sequence at least 90% identical to SEQ ID NO:8 or SEQ ID NO:10. More preferably, such a receptor SEQ ID NO:8 or SEQ ID NO:10.

According to a nineteenth aspect, the present invention provides a method of controlling gene expression in a transgenic plant, comprising: expressing in the transgenic plant a

chimeric receptor polypeptide of the invention, as described above, and a target expression cassette comprising a 5' regulatory region operatively linked to a target nucleotide sequence, wherein the 5' regulatory region comprises one or more response elements complementary to the DNA binding (C) domain of the chimeric receptor polypeptide; and contacting the transgenic plant with a chemical ligand that is complementary to the ligand binding (E) domain of the chimeric receptor polypeptide, whereby the chimeric receptor polypeptide in the presence of the chemical ligand activates expression of the target nucleotide sequence. Preferably, the ligand binding (E) domain of the chimeric receptor polypeptide is a *Manduca sexta* EcR ligand binding (E) domain. Also, preferably, the chemical ligand is tebufenozide or methoxytebufenozide.

In an especially preferred embodiment, the present invention provides a method of controlling gene expression in a transgenic plant, comprising: expressing in the transgenic plant (i) a chimeric receptor polypeptide comprising a DNA binding (C) domain, a hinge (D) domain of a *Manduca sexta* ecdysone receptor, a ligand binding (E) domain of a *Manduca sexta* ecdysone receptor, and an activation domain; and (ii) a target expression cassette comprising a 5' regulatory region operatively linked to a target nucleotide sequence, wherein the 5' regulatory region comprises one or more response elements complementary to the DNA binding (C) domain of the chimeric receptor polypeptide; and contacting the transgenic plant with a chemical ligand that is complementary to the ligand binding (E) domain of the chimeric receptor polypeptide, whereby the chimeric receptor polypeptide in the presence of the chemical ligand activates expression of the target nucleotide sequence. Preferably, the DNA binding (C) domain is a GAL4 DNA binding domain. Also, preferably, the activation domain of the chimeric receptor polypeptide is a VP16 activation domain, a maize C1 activation domain, or a maize Dof1 activation domain. Further, preferably, the chemical ligand is tebufenozide or methoxytebufenozide.

Other aspects and advantages of the present invention will become apparent to those skilled in the art from a study of the following description of the invention and non-limiting examples.

BRIEF DESCRIPTION OF THE SEQUENCES IN THE SEQUENCE LISTING

SEQ ID NO:1 shows a nucleotide sequence that encodes the ecdysone receptor of *Manduca sexta* (tobacco hornworm).

SEQ ID NO:2 shows the amino acid sequence of the *Manduca sexta* ecdysone receptor encoded by SEQ ID NO:1.

SEQ ID NO:3 shows the 5' end of a nucleotide sequence that encodes the ecdysone receptor of *Ostrinia nubilalis* (European cornborer).

SEQ ID NO:4 shows the amino acid sequence of the N-terminus of the *Ostrinia nubilalis* ecdysone receptor encoded by SEQ ID NO:3.

SEQ ID NO:5 shows the 3' end of a nucleotide sequence that encodes the ecdysone receptor of *Ostrinia nubilalis* (European cornborer).

SEQ ID NO:6 shows the amino acid sequence of the C-terminus of the *Ostrinia nubilalis* ecdysone receptor encoded by SEQ ID NO:5. SEQ ID NO:4 and SEQ ID NO:6 collectively comprise the A/B, C, D, and E domains of the *Ostrinia nubilalis* ecdysone receptor.

SEQ ID NO:7 shows the 3' end of a nucleotide sequence that encodes the ecdysone receptor of *Spodoptera frugiperda* (Fall armyworm).

SEQ ID NO:8 shows the amino acid sequence of the C-terminus (a portion of the D domain and the full E domain) of the *Spodoptera frugiperda* ecdysone receptor encoded by SEQ ID NO:7.

SEQ ID NO:9 shows the 3' end of a nucleotide sequence that encodes the ecdysone receptor of *Agrotis ipsilon* (Black cutworm).

SEQ ID NO:10 shows the amino acid sequence of the C-terminus (a portion of the D domain and the full E domain) of the *Agrotis ipsilon* ecdysone receptor encoded by SEQ ID NO:9.

SEQ ID NO:11 shows a nucleotide sequence that encodes the ecdysone receptor of *Locusta migratoria* (migratory locust).

SEQ ID NO:12 shows the amino acid sequence of the *Locusta migratoria* ecdysone receptor encoded by SEQ ID NO:11.

SEQ ID NO:13 shows a nucleotide sequence that encodes the ecdysone receptor of *Chironomus tentans*.

SEQ ID NO:14 shows the amino acid sequence of the *Chironomus tentans* ecdysone receptor encoded by SEQ ID NO:13.

SEQ ID NO:15 through SEQ ID NO:41 are oligonucleotide primers.

SEQ ID NO:42 shows the nucleotide sequence of the inserted region in pCGS154.

SEQ ID NO:43 through SEQ ID NO:60 are oligonucleotide primers.

SEQ ID NO:61 and SEQ ID NO:62 collectively show a double stranded oligonucleotide used to create a multiple cloning site (MCS), which has the recognition sequences for restriction enzymes *Sma*I, *Sal*I, *Eco*RI, *Bsp*EI, *Hind*III, and *Xba*I.

SEQ ID NO:63 shows the nucleotide sequence that encodes the ecdysone receptor chimera MDV, which comprises the *Manduca sexta* C and D domains, the *Drosophila melanogaster* E domain, and the VP16 Activation domain. Nucleotides 1-1263 code for the EcR C, D, and E domains, whereas nucleotides 1264-1506 code for the VP16 Activation domain.

SEQ ID NO:64 shows the amino acid sequence of the ecdysone receptor chimera MDV encoded by SEQ ID NO:63. Amino acids 1-421 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:65 shows the nucleotide sequence that encodes the ecdysone receptor chimera MBV, which comprises the *Manduca sexta* C and D domains, the black cutworm (*Agrotis ipsilon*) E domain, and the VP16 Activation domain. Nucleotides 1-1266 code for the EcR C, D, and E domains, whereas nucleotides 1267-1509 code for the VP16 Activation domain.

SEQ ID NO:66 shows the amino acid sequence of the ecdysone receptor chimera MBV encoded by SEQ ID NO:65. Amino acids 1-422 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:67 shows the nucleotide sequence that encodes the ecdysone receptor chimera MEV, which comprises the *Manduca sexta* C and D domains, the European corn borer (*Ostrinia nubilalis*) E domain, and the VP16 Activation domain. Nucleotides 1-1257 code for the EcR C, D, and E domains, whereas nucleotides 1258-1500 code for the VP16 Activation domain.

SEQ ID NO:68 shows the amino acid sequence of the ecdysone receptor chimera MEV encoded by SEQ ID NO:67. Amino acids 1-419 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:69 shows the nucleotide sequence that encodes the ecdysone receptor chimera MFV, which comprises the *Manduca sexta* C and D domains, the fall armyworm (*Spodoptera frugiperda*) E domain, and the VP16 Activation domain. Nucleotides 1-1257 code for the EcR C, D, and E domains, whereas nucleotides 1258-1500 code for the VP16 Activation domain.

SEQ ID NO:70 shows the amino acid sequence of the ecdysone receptor chimera MFV encoded by SEQ ID NO:69. Amino acids 1-419 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:71 shows the nucleotide sequence that encodes the ecdysone receptor chimera DMV, which comprises the *Drosophila melanogaster* C and D domains, the *Manduca sexta* E domain, and the VP16 Activation domain. Nucleotides 1-1308 code for the EcR C, D, and E domains, whereas nucleotides 1309-1551 code for the VP16 Activation domain.

SEQ ID NO:72 shows the amino acid sequence of the ecdysone receptor chimera DMV encoded by SEQ ID NO:71. Amino acids 1-436 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:73 shows the nucleotide sequence that encodes the ecdysone receptor chimera DBV, which comprises the *Drosophila melanogaster* C and D domains, the black cutworm (*Agrotis ipsilon*) E domain, and the VP16 Activation domain. Nucleotides 1-1299 code for the EcR C, D, and E domains, whereas nucleotides 1300-1542 code for the VP16 Activation domain.

SEQ ID NO:74 shows the amino acid sequence of the ecdysone receptor chimera DBV encoded by SEQ ID NO:73. Amino acids 1-433 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:75 shows the nucleotide sequence that encodes the ecdysone receptor chimera EEV, which comprises the European corn borer (*Ostrinia nubilalis*) C and D domains, the European corn borer E domain, and the VP16 Activation domain. Nucleotides 1-

1272 code for the EcR C, D, and E domains, whereas nucleotides 1273-1515 code for the VP16 Activation domain.

SEQ ID NO:76 shows the amino acid sequence of the ecdysone receptor chimera EEV encoded by SEQ ID NO:75. Amino acids 1-424 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:77 shows the nucleotide sequence that encodes the ecdysone receptor chimera EBV, which comprises the European corn borer (*Ostrinia nubilalis*) C and D domains, the black cutworm (*Agrotis ipsilon*) E domain, and the VP16 Activation domain. Nucleotides 1-1281 code for the EcR C, D, and E domains, whereas nucleotides 1282-1524 code for the VP16 Activation domain.

SEQ ID NO:78 shows the amino acid sequence of the ecdysone receptor chimera EBV encoded by SEQ ID NO:77. Amino acids 1-427 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:79 shows the nucleotide sequence that encodes the ecdysone receptor chimera EMV, which comprises the European corn borer (*Ostrinia nubilalis*) C and D domains, the *Manduca sexta* E domain, and the VP16 Activation domain. Nucleotides 1-1290 code for the EcR C, D, and E domains, whereas nucleotides 1291-1533 code for the VP16 Activation domain.

SEQ ID NO:80 shows the amino acid sequence of the ecdysone receptor chimera EMV encoded by SEQ ID NO:79. Amino acids 1-430 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:81 shows the nucleotide sequence that encodes the ecdysone receptor chimera LLV, which comprises the *Locusta migratoria* C and D domains, the *Locusta migratoria* E domain, and the VP16 Activation domain. Nucleotides 1-1194 code for the EcR C, D, and E domains, whereas nucleotides 1195-1437 code for the VP16 Activation domain.

SEQ ID NO:82 shows the amino acid sequence of the ecdysone receptor chimera LLV encoded by SEQ ID NO:81. Amino acids 1-398 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:83 shows the nucleotide sequence that encodes the ecdysone receptor chimera LMV, which comprises the *Locusta migratoria* C and D domains, the *Manduca sexta*

E domain, and the VP16 Activation domain. Nucleotides 1-1221 code for the EcR C, D, and E domains, whereas nucleotides 1222-1464 code for the VP16 Activation domain.

SEQ ID NO:84 shows the amino acid sequence of the ecdysone receptor chimera LMV encoded by SEQ ID NO:83. Amino acids 1-407 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:85 shows the nucleotide sequence that encodes the ecdysone receptor chimera MLV, which comprises the *Manduca sexta* C and D domains, the *Locusta migratoria* E domain, and the VP16 Activation domain. Nucleotides 1-1248 code for the EcR C, D, and E domains, whereas nucleotides 1249-1491 code for the VP16 Activation domain.

SEQ ID NO:86 shows the amino acid sequence of the ecdysone receptor chimera MLV encoded by SEQ ID NO:85. Amino acids 1-416 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:87 shows the nucleotide sequence that encodes the ecdysone receptor chimera CCV, which comprises the *Chironomus tentans* C and D domains, the *Chironomus tentans* E domain, and the VP16 Activation domain. Nucleotides 1-1308 code for the EcR C, D, and E domains, whereas nucleotides 1309-1551 code for the VP16 Activation domain.

SEQ ID NO:88 shows the amino acid sequence of the ecdysone receptor chimera CCV encoded by SEQ ID NO:87. Amino acids 1-436 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:89 shows the nucleotide sequence that encodes the ecdysone receptor chimera CMV, which comprises the *Chironomus tentans* C and D domains, the *Manduca sexta* E domain, and the VP16 Activation domain. Nucleotides 1-1323 code for the EcR C, D, and E domains, whereas nucleotides 1324-1566 code for the VP16 Activation domain.

SEQ ID NO:90 shows the amino acid sequence of the ecdysone receptor chimera CMV encoded by SEQ ID NO:89. Amino acids 1-441 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:91 shows the nucleotide sequence that encodes the ecdysone receptor chimera MCV, which comprises the *Manduca sexta* C and D domains, the *Chironomus tentans* E domain, and the VP16 Activation domain. Nucleotides 1-1260 code for the EcR C, D, and E domains, whereas nucleotides 1261-1503 code for the VP16 Activation domain.

SEQ ID NO:92 shows the amino acid sequence of the ecdysone receptor chimera MCV encoded by SEQ ID NO:91. Amino acids 1-420 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:93 shows the nucleotide sequence that encodes the ecdysone receptor chimera MMV, which comprises the *Manduca sexta* C and D domains, the *Manduca sexta* E domain, and the VP16 Activation domain. Nucleotides 1-1275 code for the EcR C, D, and E domains, whereas nucleotides 1276-1518 code for the VP16 Activation domain.

SEQ ID NO:94 shows the amino acid sequence of the ecdysone receptor chimera MMV encoded by SEQ ID NO:93. Amino acids 1-425 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:95 shows the nucleotide sequence that encodes the ecdysone receptor chimera DDV, which comprises the *Drosophila melanogaster* C and D domains, the *Drosophila melanogaster* E domain, and the VP16 Activation domain. Nucleotides 1-1296 code for the EcR C, D, and E domains, whereas nucleotides 1297-1539 code for the VP16 Activation domain.

SEQ ID NO:96 shows the amino acid sequence of the ecdysone receptor chimera DDV encoded by SEQ ID NO:95. Amino acids 1-432 constitute the C, D, and E domains of the receptor chimera.

SEQ ID NO:97 through SEQ ID NO:102 are oligonucleotide primers.

SEQ ID NO:103 shows the nucleotide sequence of the reporter fragment cloned into pCGS601.

SEQ ID NO:104 shows the nucleotide sequence (nucleotides 2007-3668) that encodes the ecdysone receptor chimera G(M)MV, which comprises the GAL4 DNA Binding Domain, the *Manduca* D and E Domains, and the VP16 Activation Domain, as contained in pCGS202.

SEQ ID NO:105 shows the amino acid sequence of the ecdysone receptor chimera G(M)MV encoded by nucleotides 2007-3668 of SEQ ID NO:104. Amino acids 1-147 constitute the GAL4 DNA Binding Domain, amino acids 148-473 constitute the *Manduca* D and E Domains, and amino acids 474-553 constitute the VP16 Activation domain.

SEQ ID NO:106 shows the nucleotide sequence of the maize Adh intron number 1.

SEQ ID NO:107 shows the nucleotide sequence of the maize shrunken (Sh) intron number 1.

SEQ ID NO:108 shows the nucleotide sequence of the maize ubiquitin intron number 1

SEQ ID NO:109 shows the nucleotide sequence of the rice actin intron.

SEQ ID NO:110 through SEQ ID NO:117 are oligonucleotide primers.

SEQ ID NO:118 shows the nucleotide sequence that encodes the ecdysone receptor chimera G(M)BV, which comprises the GAL4 DNA Binding Domain, the *Manduca* D and E Domains, and the VP16 Activation Domain. Nucleotides 1-564 code for the GAL4 DNA Binding Domain; nucleotides 565-848 code for the *Manduca* hinge (D) domain; nucleotides 849-1533 code for the BCW (*Agrotis ipsilon*) ligand binding (E) domain; and nucleotides 1534-1776 code for the VP16 Activation Domain.

SEQ ID NO:119 shows the amino acid sequence of the ecdysone receptor chimera G(M)BV encoded by SEQ ID NO:118.

SEQ ID NO:120 shows the nucleotide sequence that encodes the ecdysone receptor chimera G(M)EV, which comprises the GAL4 DNA Binding Domain, the *Manduca* D Domain, the ECB (*Ostrinia nubilalis*) Ligand Binding (E) Domain, and the VP16 Activation Domain. Nucleotides 1-564 code for the GAL4 DNA Binding Domain; nucleotides 565-848 code for the *Manduca* hinge (D) domain; nucleotides 849-1524 code for the ECB (*Ostrinia nubilalis*) ligand binding (E) domain; and nucleotides 1525-1767 code for the VP16 Activation Domain.

SEQ ID NO:121 shows the amino acid sequence of the ecdysone receptor chimera G(M)EV encoded by SEQ ID NO:120.

SEQ ID NO:122 shows the nucleotide sequence that encodes the ecdysone receptor chimera G(M)FV, which comprises the GAL4 DNA Binding Domain, the *Manduca* D Domain, the FAW (*Spodoptera frugiperda*) Ligand Binding (E) Domain, and the VP16 Activation Domain. Nucleotides 1-564 code for the GAL4 DNA Binding Domain; nucleotides 565-848 code for the *Manduca* hinge (D) domain; nucleotides 849-1524 code for the FAW (*Spodoptera frugiperda*) ligand binding (E) domain; and nucleotides 1525-1767 code for the VP16 Activation Domain.

SEQ ID NO:123 shows the amino acid sequence of the ecdysone receptor chimera G(M)FV encoded by SEQ ID NO:122.

SEQ ID NO:124 shows the nucleotide sequence that encodes the ecdysone receptor chimera G(E)EV, which comprises the GAL4 DNA Binding Domain, the ECB (*Ostrinia*

nubilalis) D Domain, the ECB (*Ostrinia nubilalis*) Ligand Binding (E) Domain, and the VP16 Activation Domain. Nucleotides 1-564 code for the GAL4 DNA Binding Domain; nucleotides 565-863 code for the ECB (*Ostrinia nubilalis*) hinge (D) domain; nucleotides 864-1539 code for the ECB (*Ostrinia nubilalis*) ligand binding (E) domain; and nucleotides 1540-1782 code for the VP16 Activation Domain.

SEQ ID NO:125 shows the amino acid sequence of the ecdysone receptor chimera G(E)EV encoded by SEQ ID NO:124.

SEQ ID NO:126 shows the nucleotide sequence that encodes the ecdysone receptor chimera G(E)MV, which comprises the GAL4 DNA Binding Domain, the ECB (*Ostrinia nubilalis*) D Domain, the *Manduca* Ligand Binding (E) Domain, and the VP16 Activation Domain. Nucleotides 1-564 code for the GAL4 DNA Binding Domain; nucleotides 565-863 code for the ECB (*Ostrinia nubilalis*) hinge (D) domain; nucleotides 864-1557 code for the *Manduca* ligand binding (E) domain; and nucleotides 1558-1800 code for the VP16 Activation Domain.

SEQ ID NO:127 shows the amino acid sequence of the ecdysone receptor chimera G(E)MV encoded by SEQ ID NO:126.

SEQ ID NO:128 shows the G(M)M (GAL4 DNA Binding Domain fused to the *Manduca* EcR Hinge and Ligand Binding Domain) chimeric receptor nucleotide coding sequence.

SEQ ID NO:129 shows the amino acid sequence of the GAL4 DNA Binding Domain fused to the *Manduca* EcR Hinge and Ligand Binding Domain encoded by SEQ ID NO:128.

SEQ ID NO:130 through SEQ ID NO:133 are oligonucleotide primers.

SEQ ID NO:134 shows the nucleotide sequence that encodes the ecdysone receptor chimera G(M)MC, which comprises the GAL4 DNA Binding Domain, the *Manduca* D Domain, the *Manduca* Ligand Binding (E) Domain, and the maize C1 Activation Domain.

SEQ ID NO:135 shows the amino acid sequence of the ecdysone receptor chimera G(M)MC encoded by SEQ ID NO:134.

SEQ ID NO:136 shows the nucleotide sequence that encodes the ecdysone receptor chimera G(M)MD, which comprises the GAL4 DNA Binding Domain, the *Manduca* D Domain, the *Manduca* Ligand Binding (E) Domain, and the maize Dof1 Activation Domain.

SEQ ID NO:137 shows the amino acid sequence of the ecdysone receptor chimera G(M)MD encoded by SEQ ID NO:136.

SEQ ID NO:138 through SEQ ID NO:141 are oligonucleotide primers.

SEQ ID NO:142 shows the nucleotide sequence that encodes the ecdysone receptor chimera VG(M)M, which comprises the VP16 Activation Domain, the GAL4 DNA Binding Domain, the *Manduca* D Domain, and the *Manduca* Ligand Binding (E) Domain.

SEQ ID NO:143 shows the amino acid sequence of the ecdysone receptor chimera VG(M)M encoded by SEQ ID NO:142.

SEQ ID NO:144 through SEQ ID NO:146 are oligonucleotide primers.

SEQ ID NO:147 shows the nucleotide sequence that encodes the ecdysone receptor chimera GV(M)M, which comprises the GAL4 DNA Binding Domain, the VP16 Activation Domain, the *Manduca* D Domain, and the *Manduca* Ligand Binding (E) Domain.

SEQ ID NO:148 shows the amino acid sequence of the ecdysone receptor chimera GV(M)M encoded by SEQ ID NO:147.

DEFINITIONS

"Associated with / operatively linked" refer to two nucleic acid sequences that are related physically or functionally. For example, a promoter or regulatory DNA sequence is said to be "associated with" a DNA sequence that codes for an RNA or a protein if the two sequences are operatively linked, or situated such that the regulator DNA sequence will affect the expression level of the coding or structural DNA sequence.

A "chimeric" gene is a recombinant nucleic acid sequence in which a promoter or regulatory nucleic acid sequence is operatively linked to, or associated with, a nucleic acid sequence that codes for an mRNA or which is expressed as a protein, such that the regulator nucleic acid sequence is able to regulate transcription or expression of the associated nucleic acid sequence. The regulator nucleic acid sequence of the chimeric gene is not normally operatively linked to the associated nucleic acid sequence as found in nature.

In the context of the present invention, the term "chimeric" is also used to indicate that the receptor polypeptide is comprised of domains, at least one of which has an origin that is heterologous with respect to the other domains present. These chimeric receptor polypeptides

are encoded by nucleotide sequences that have been fused or ligated together resulting in a coding sequence that does not occur naturally.

Chimeric receptor polypeptides of the present invention are referenced by a linear nomenclature from N-terminal to C-terminal portion of the polypeptide. Using this nomenclature, a chimeric receptor polypeptide having the transactivation domain from VP16 fused to the N-terminal end of the EcR receptor would be designated as VP16-EcR. Conversely, if VP16 is fused to the C-terminus of the EcR receptor, the chimeric receptor polypeptide would be designated EcR-VP16.

Chimeric receptor polypeptides of the present invention are alternately referenced by a linear triplet or linear quartet nomenclature to indicate which domains are present in the construct in N-terminal to C-terminal orientation. In the case of triplet nomenclature, the first letter of the triplet corresponds to the origin of the DNA binding and hinge domains; the second letter of the triplet indicates the origin of the ligand binding domain; and the last letter of the triplet indicates the activation domain. In this manner, the chimeric receptors are designated in the table set forth in Example 5. For example, the MDV EcR chimera comprises the DNA binding and hinge domains from *Manduca sexta*, the ligand binding domain from *Drosophila melanogaster* and the activation domain of VP16; and the MBV EcR chimera comprises the DNA binding and hinge domains from *Manduca sexta*, the ligand binding domain from *Agrotis ipsilon* (Black cutworm) and the activation domain of VP16.

In the case of quartet nomenclature, the first letter of the quartet corresponds to the origin of the DNA binding domain; the second letter of the quartet (which is in brackets) corresponds to the origin of the hinge domain; the third letter of the quartet indicates the origin of the ligand binding domain; and the last letter of the quartet indicates the activation domain. In this manner, the chimeric receptors are designated in the table set forth in Example 23. For example, the G(M)EV EcR chimera comprises the yeast GAL4 DNA binding domain, the hinge domain from *Manduca sexta*, the ligand binding domain from European corn borer (*Ostrinia nubilalis*), and the VP16 activation domain; and the G(M)MV EcR chimera comprises the yeast GAL4 DNA binding domain, the hinge domain from *Manduca sexta*, the ligand binding domain from *Manduca sexta*, and the VP16 activation domain.

Gene constructions are denominated in terms of a 5' regulatory region and its operably-linked coding sequence, where the 5' regulatory region is designated before a slash mark (/) and the coding sequence designated after the slash mark. For example, the gene construction ubi/EcR-VP16 designates the ubiquitin promoter (of e.g. *Zea mays*) fused to the chimeric receptor EcR-VP16, where the transactivation domain of VP16 is fused to the C-terminal end of EcR.

A "coding sequence" is a nucleic acid sequence that is transcribed into RNA such as mRNA, rRNA, tRNA, snRNA, sense RNA or antisense RNA. Preferably the RNA is then translated in an organism to produce a protein.

Complementary: "complementary" refers to two nucleotide sequences that comprise antiparallel nucleotide sequences capable of pairing with one another upon formation of hydrogen bonds between the complementary base residues in the antiparallel nucleotide sequences.

"Conservatively modified variations" of a particular nucleic acid sequence refers to those nucleic acid sequences that encode identical or essentially identical amino acid sequences, or where the nucleic acid sequence does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance the codons CGT, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded protein. Such nucleic acid variations are "silent variations" which are one species of "conservatively modified variations." Every nucleic acid sequence described herein which encodes a protein also describes every possible silent variation, except where otherwise noted. One of skill will recognize that each codon in a nucleic acid (except ATG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a protein is implicit in each described sequence.

Furthermore, one of skill will recognize that individual substitutions deletions or additions that alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are

"conservatively modified variations," where the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following five groups each contain amino acids that are conservative substitutions for one another: Aliphatic: Glycine (G), Alanine (A), Valine (V), Leucine (L), Isoleucine (I); Aromatic: Phenylalanine (F), Tyrosine (Y), Tryptophan (W); Sulfur-containing: Methionine (M), Cysteine (C); Basic: Arginine (R), Lysine (K), Histidine (H); Acidic: Aspartic acid (D), Glutamic acid (E), Asparagine (N), Glutamine (Q). *See also,* Creighton (1984) *Proteins, W.H.* Freeman and Company. In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservatively modified variations."

A "DNA binding domain" (a.k.a. "C domain") is the portion of a receptor polypeptide that comprises a sequence of amino acids that binds non-covalently a specific nucleotide sequence known as a response element (RE). In nuclear receptors, response elements are located in the 5' regulatory region of a target expression cassette and comprise a pair of half-sites, each half-site having a 6 base pair core wherein a single DNA binding domain recognizes a single half-site. The half-sites may be arranged in relative linear orientation to each other as either direct repeats, palindromic repeats, or inverted repeats. A response element binds either a homodimer or heterodimer of receptor polypeptides. The nucleotide sequence and linear orientation of the half-sites determines which DNA binding domain or domains will form a complementary binding pair with said response element, as well as the ability of receptor polypeptides to interact with each other in a dimer.

"Ecdysone receptor" ("EcR") refers to the receptors found in certain insects that are known to bind ecdysone as their ligand or that have high homology with previously isolated ecdysone receptors from other insects. Ecdysone receptors have been isolated from a number of insects including dipteran, coleopteran, and lepidopteran insects.

"Expression cassette" as used herein means a nucleic acid sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operatively linked to the nucleotide sequence of interest, which is operatively linked to termination signals. It also typically comprises sequences required for proper translation of the nucleotide sequence. The expression cassette comprising the

nucleotide sequence of interest may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. The expression cassette may also be one that is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. Typically, however, the expression cassette is heterologous with respect to the host, i.e., the particular nucleic acid sequence of the expression cassette does not occur naturally in the host cell and has been introduced into the host cell or an ancestor of the host cell by a transformation event. The expression of the nucleotide sequence in the expression cassette may be under the control of either a constitutive promoter or an inducible promoter that initiates transcription only when the host cell is exposed to some particular external stimulus. In the case of a multicellular organism, such as a plant, the promoter can also be specific to a particular tissue, or organ, or stage of development.

Gene: the term "gene" is used broadly to refer to any segment of DNA associated with a biological function. Thus, genes include coding sequences and/or the regulatory sequences required for their expression. Genes also include nonexpressed DNA segments that, for example, form recognition sequences for other proteins. Genes can be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information, and may include sequences designed to have desired parameters.

"Gene of interest" refers to any gene which, when transferred to a host organism, confers upon the host a desired characteristic. In the case of plant hosts, desired characteristics include antibiotic resistance, virus resistance, insect resistance, disease resistance, or resistance to other pests, herbicide tolerance, improved nutritional value, improved performance in an industrial process or altered reproductive capability. The "gene of interest" may also be one that is transferred to a host organism, e.g. a plant, for the production of commercially valuable enzymes or metabolites in the host.

Heterologous/exogenous: The terms "heterologous" and "exogenous" when used herein to refer to a nucleic acid sequence (e.g. a DNA sequence) or a gene, refer to a sequence that originates from a source foreign to the particular host cell or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell but has been modified through, for example, the use of codon optimization. The terms also includes non-naturally occurring multiple copies of a

naturally occurring sequence. Thus, the terms refer to a nucleic acid segment that is foreign or heterologous to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is not ordinarily found. Exogenous nucleic acid segments are expressed to yield exogenous polypeptides. For example, in the context of the present invention, "heterologous" is used to indicate that a receptor polypeptide has a different natural origin with respect to its current host. For example, if the ecdysone receptor (EcR) from an insect species is expressed in a plant cell, then the EcR is described as being heterologous with respect to its current host, which is the plant cell. "Heterologous" is also used to indicate that one or more of the domains present in a receptor polypeptide differ in their natural origin with respect to other domains present. For example, if the transactivation domain from the herpes simplex VP16 protein is fused to the ecdysone receptor from *Manduca sexta*, then the VP16 transactivation domain is heterologous with respect to the EcR-moiety. Furthermore, if a domain from *Manduca sexta* EcR is fused to a domain from *Agrotis ipsilon* EcR to make a functional receptor, then the chimeric fusion would have domains that are heterologous with respect to each other. In addition, a heterologous receptor polypeptide comprising the fusion of the VP16 protein to the *Manduca sexta* ecdysone receptor, when expressed in a plant, would also be considered heterologous with respect to the plant host.

A "hinge domain" (a.k.a. "D domain") is the portion of a receptor polypeptide that comprises the amino acids between the DNA binding (C) domain and ligand binding (E) domain. The hinge domain may participate in the interaction of the receptor polypeptide with another receptor polypeptide to form either a homodimer or heterodimer.

A "homologous" nucleic acid (e.g. DNA) sequence is a nucleic acid (e.g. DNA) sequence naturally associated with a host cell into which it is introduced. For example, in the context of the present invention, "homologous" is used to indicate that a receptor polypeptide has the same natural origin with respect to its current host. For example, the ecdysone receptor is found in certain insect species and is said to be homologous with respect to the insect species in which it originates. "Homologous" is also used to indicate that one or more of the domains present in a receptor polypeptide have the same natural origin with respect to each other. For example, the DNA binding domain and the ligand binding domain of *Manduca sexta* EcR are considered to be of a homologous origin with respect to each other.

"Homologous recombination" is the reciprocal exchange of nucleic acid fragments between homologous nucleic acid molecules.

The terms "identical" or percent "identity" in the context of two or more nucleic acid or protein sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the sequence comparison algorithms described below or by visual inspection.

A nucleic acid sequence is "isocoding with" a reference nucleic acid sequence when the nucleic acid sequence encodes a polypeptide having the same amino acid sequence as the polypeptide encoded by the reference nucleic acid sequence.

An "isolated" nucleic acid molecule or an isolated enzyme is a nucleic acid molecule or enzyme that, by the hand of man, exists apart from its native environment and is therefore not a product of nature. An isolated nucleic acid molecule or enzyme may exist in a purified form or may exist in a non-native environment such as, for example, a recombinant host cell.

A "ligand binding domain" (a.k.a. "E domain") is the portion of a receptor polypeptide that comprises a sequence of amino acids whose structure binds non-covalently a chemical ligand. Hence, a ligand binding domain and its chemical ligand form a binding pair.

Mature Protein: protein that is normally targeted to a cellular organelle and from which the transit peptide has been removed.

Minimal Promoter: promoter elements, particularly a TATA element, that are inactive or that have greatly reduced promoter activity in the absence of upstream activation. In the presence of a suitable transcription factor, the minimal promoter functions to permit transcription.

A "moiety" refers to that share or portion of a receptor polypeptide that is derived from the indicated source. For example, "EcR-moiety" refers to that portion of the receptor polypeptide that was derived from a native ecdysone receptor. A "moiety" as used herein may comprise one or more domains.

Native: refers to a gene that is present in the genome of an untransformed cell.

Naturally occurring: the term "naturally occurring" is used to describe an object that can be found in nature as distinct from being artificially produced by man. For example, a protein or nucleotide sequence present in an organism (including a virus), which can be

isolated from a source in nature and which has not been intentionally modified by man in the laboratory, is naturally occurring.

Nucleic acid: the term "nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof *(e.g.* degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al., Nucleic Acid Res.* 19: 5081 (1991); Ohtsuka *et al., J. Biol. Chem.* 260: 2605-2608 (1985); Rossolini *et al., Mol. Cell. Probes* 8: 91-98 (1994)). The terms "nucleic acid" or "nucleic acid sequence" may also be used interchangeably with gene, cDNA, and mRNA encoded by a gene.

"ORF" means open reading frame.

A "plant" is any plant at any stage of development, particularly a seed plant.

A "plant cell" is a structural and physiological unit of a plant, comprising a protoplast and a cell wall. The plant cell may be in form of an isolated single cell or a cultured cell, or as a part of higher organized unit such as, for example, plant tissue, a plant organ, or a whole plant.

"Plant cell culture" means cultures of plant units such as, for example, protoplasts, cell culture cells, cells in plant tissues, pollen, pollen tubes, ovules, embryo sacs, zygotes and embryos at various stages of development.

"Plant material" refers to leaves, stems, roots, flowers or flower parts, fruits, pollen, egg cells, zygotes, seeds, cuttings, cell or tissue cultures, or any other part or product of a plant.

A "plant organ" is a distinct and visibly structured and differentiated part of a plant such as a root, stem, leaf, flower bud, or embryo.

"Plant tissue" as used herein means a group of plant cells organized into a structural and functional unit. Any tissue of a plant *in planta* or in culture is included. This term

includes, but is not limited to, whole plants, plant organs, plant seeds, tissue culture and any groups of plant cells organized into structural and/or functional units. The use of this term in conjunction with, or in the absence of, any specific type of plant tissue as listed above or otherwise embraced by this definition is not intended to be exclusive of any other type of plant tissue.

A "promoter" is an untranslated DNA sequence upstream of the coding region that contains the binding site for RNA polymerase II and initiates transcription of the DNA. The promoter region may also include other elements that act as regulators of gene expression.

A "protoplast" is an isolated plant cell without a cell wall or with only parts of the cell wall.

Purified: the term "purified," when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein which is the predominant species present in a preparation is substantially purified. The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least about 50% pure, more preferably at least about 85% pure, and most preferably at least about 99% pure.

A "receptor cassette" as used herein comprises a nucleotide sequence that encodes a receptor polypeptide.

A "receptor expression cassette" as used herein comprises a nucleotide sequence for a 5' regulatory region, e.g. a promoter that permits expression in plant tissues, operatively linked to a nucleotide sequence that encodes a receptor polypeptide and an untranslated 3' termination region (stop codon and polyadenylation sequence).

"Receptor polypeptide" as used herein refers to a polypeptide that activates the expression of a target gene of interest or target expression cassette in response to an applied chemical ligand. The receptor polypeptide is comprised of a ligand binding domain, a DNA binding domain and a transactivation domain.

Two nucleic acids are "recombined" when sequences from each of the two nucleic acids are combined in a progeny nucleic acid. Two sequences are "directly" recombined when both of the nucleic acids are substrates for recombination. Two sequences are "indirectly recombined" when the sequences are recombined using an intermediate such as a cross-over oligonucleotide. For indirect recombination, no more than one of the sequences is an actual substrate for recombination, and in some cases, neither sequence is a substrate for recombination.

"Regulatory elements" refer to sequences involved in controlling the expression of a nucleotide sequence. Regulatory elements comprise a promoter operatively linked to the nucleotide sequence of interest and termination signals. They also typically encompass sequences required for proper translation of the nucleotide sequence.

Substantially identical: the phrase "substantially identical," in the context of two nucleic acid or protein sequences, refers to two or more sequences or subsequences that have at least 60%, preferably 80%, more preferably 90, even more preferably 95%, and most preferably at least 99% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. Preferably, the substantial identity exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over at least about 150 residues. In an especially preferred embodiment, the sequences are substantially identical over the entire length of the coding regions. Furthermore, substantially identical nucleic acid or protein sequences perform substantially the same function.

For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48: 443 (1970), by the search for

similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (*see generally,* Ausubel *et al., infra).*

One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al., J. Mol. Biol.* 215: 403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, 1990). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity X from its maximum achieved value, the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments, or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix *(see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89: 10915 (1989)).

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences *(see, e.g.,* Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90: 5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an

indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a test nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid sequence to the reference nucleic acid sequence is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions. The phrase "hybridizing specifically to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (*e.g.*, total cellular) DNA or RNA. "Bind(s) substantially" refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target nucleic acid sequence.

"Stringent hybridization conditions" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and Northern hybridizations are sequence dependent, and are different under different environmental parameters. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic* Acid Probes part I chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays" Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. Typically, under "stringent conditions" a probe will hybridize to its target subsequence, but to no other sequences.

The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formamide with 1 mg of heparin at 42°C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.1 5M NaCl at 72°C for about 15 minutes. An example of stringent wash

conditions is a 0.2x SSC wash at 65°C for 15 minutes (*see,* Sambrook, *infra*, for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1x SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, *e.g.,* more than 100 nucleotides, is 4-6x SSC at 40°C for 15 minutes. For short probes (*e.g.,* about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30°C. Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the proteins that they encode are substantially identical. This occurs, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

The following are examples of sets of hybridization/wash conditions that may be used to clone homologous nucleotide sequences that are substantially identical to reference nucleotide sequences of the present invention: a reference nucleotide sequence preferably hybridizes to the reference nucleotide sequence in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 2X SSC, 0.1% SDS at 50°C, more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 1X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.5X SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 65°C.

A further indication that two nucleic acid sequences or proteins are substantially identical is that the protein encoded by the first nucleic acid is immunologically cross reactive with, or specifically binds to, the protein encoded by the second nucleic acid. Thus, a protein is typically substantially identical to a second protein, for example, where the two proteins differ only by conservative substitutions.

The phrase "specifically (or selectively) binds to an antibody," or "specifically (or selectively) immunoreactive with," when referring to a protein or peptide, refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, antibodies raised to the protein with the amino acid sequence encoded by any of the nucleic acid sequences of the invention can be selected to obtain antibodies specifically immunoreactive with that protein and not with other proteins except for polymorphic variants. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays, Western blots, or immunohistochemistry are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) *Antibodies, A Laboratory Manual,* Cold Spring Harbor Publications, New York "Harlow and Lane"), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity. Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 to 100 times background.

A "subsequence" refers to a sequence of nucleic acids or amino acids that comprise a part of a longer sequence of nucleic acids or amino acids (e.g., protein) respectively.

"Synthetic" refers to a nucleotide sequence comprising structural characters that are not present in the natural sequence. For example, an artificial sequence that resembles more closely the G+C content and the normal codon distribution of dicot and/or monocot genes is said to be synthetic.

A "target expression cassette" comprises a nucleotide sequence for a 5' regulatory region operatively linked to a target nucleotide sequence, the expression of which is activated by a receptor polypeptide in the presence of a chemical ligand. The 5' regulatory region of the target gene comprises a core promoter sequence, an initiation of transcription sequence and the one or more response elements necessary for complementary binding of the DNA binding domain of the receptor polypeptide. The promoter sequence may be a minimal promoter. The

target expression cassette also possesses a 3' termination region (stop codon and polyadenylation sequence). The target nucleotide sequence may encode a polypeptide or expression of the target nucleotide sequence may result in an RNA species that itself is active, such as an antisense RNA or a double-stranded RNA molecule.

A "transcriptional activation domain" or "transactivation domain" or "activation domain" (a.k.a. "A/B domain") is the portion of a receptor polypeptide that comprises one or more sequences of amino acids acting as subdomains that affect the operation of transcription factors during preinitiation and assembly at the TATA box. The effect of the transactivation domain is to allow repeated transcription initiation events, leading to greater levels of gene expression.

"Transformation" is a process for introducing heterologous nucleic acid into a host cell or organism. In particular, "transformation" means the stable integration of a DNA molecule into the genome of an organism of interest. Transformed cells, tissues, or insects are understood to encompass not only the end product of a transformation process, but also transgenic progeny thereof.

"Transformed / transgenic / recombinant" refer to a host organism such as a bacterium or a plant into which a heterologous nucleic acid molecule has been introduced. The nucleic acid molecule can be stably integrated into the genome of the host or the nucleic acid molecule can also be present as an extrachromosomal molecule. Such an extrachromosomal molecule can be auto-replicating. Transformed cells, tissues, or plants are understood to encompass not only the end product of a transformation process, but also transgenic progeny thereof. A "non-transformed", "non-transgenic", or "non-recombinant" host refers to a wild-type organism, e.g., a bacterium or plant, which does not contain the heterologous nucleic acid molecule.

Nucleotides are indicated by their bases by the following standard abbreviations: adenine (A), cytosine (C), thymine (T), and guanine (G). Amino acids are likewise indicated by the following standard abbreviations: alanine (Ala; A), arginine (Arg; R), asparagine (Asn; N), aspartic acid (Asp; D), cysteine (Cys; C), glutamine (Gln; Q), glutamic acid (Glu; E), glycine (Gly; G), histidine (His; H), isoleucine (Ile; I), leucine (Leu; L), lysine (Lys; K), methionine (Met; M), phenylalanine (Phe; F), proline (Pro; P), serine (Ser; S), threonine (Thr;

T), tryptophan (Trp; W), tyrosine (Tyr; Y), and valine (Val; V). Furthermore, (Xaa; X) represents any amino acid.

DETAILED DESCRIPTION OF THE INVENTION

I. Native and Chimeric Receptor Polypeptides

The present invention comprises a receptor cassette encoding a receptor polypeptide. In a preferred embodiment, the receptor polypeptide is composed of a hinge region, a ligand binding domain, a DNA binding domain, and a transactivation domain. The DNA binding domain binds the receptor polypeptide to the 5' regulatory region of a target expression cassette at the site of its response element. The hinge domain of the receptor polypeptide resides between the DNA binding and ligand binding domains and influences the activity of the ligand binding domain. The ligand binding domain of the receptor polypeptide binds, when present, a complementary chemical ligand. Binding of the chemical ligand causes a conformational change in the receptor polypeptide and allows the transactivation domain to affect transcription of the target nucleotide sequence, resulting in production of, e.g., an encoded polypeptide, an antisense RNA, or a double-stranded RNA molecule.

The chimeric receptor polypeptides used in the present invention may have one or more domains obtained from a heterologous source. The use of chimeric receptor polypeptides has the benefit of combining domains from different sources, thus providing a receptor polypeptide activated by a choice of chemical ligands and possessing desirable ligand binding, DNA binding and transactivation characteristics.

Chimeric receptor polypeptides may be used in the present invention to activate expression of a target nucleotide sequence that, e.g., encodes a target polypeptide. One or more of the four domains of a receptor polypeptide may be chosen from a heterologous source based upon their effectiveness for transactivation, DNA binding, or chemical ligand binding. The DNA binding (C) and transactivation (A/B) domains of the chimeric receptor polypeptide may also be obtained from any organism, such as plants, insects and mammals, which has similar transcriptional regulating functions. The hinge (D) and ligand binding (E) domains of the chimeric receptor polypeptide are preferably chosen from insect ecdysone receptors. In

one embodiment of the invention, the hinge (D) and ligand binding (E) domains are each selected from the ecdysone receptor of a different insect. Chimeric receptor polypeptides as provided herein offer the advantage of combining optimum transactivating activity, complementary binding of a selected chemical ligand, and recognition of a specific response element. Thus, a chimeric polypeptide may be constructed that is tailored for a specific purpose. These chimeric receptor polypeptides also provide improved functionality.

It is also considered a part of the present invention that the transactivation (A/B), ligand-binding (E), and DNA-binding (C) domains may be assembled in the chimeric receptor polypeptide in any functional arrangement. For example, where one subdomain of a transactivation domain is found at the N-terminal portion of a naturally-occuring receptor, the chimeric receptor polypeptide of the present invention may include a transactivation domain at the C-terminus in place of, or in addition to, a transactivation domain at the N-terminus. Chimeric receptor polypeptides as disclosed herein may also have multiple domains of the same type, for example, more than one transactivation domain per receptor polypeptide.

Chimeric receptor casettes and chimeric receptor polypeptides may be constructed from domains available from ecdysone receptors of the natural insect population. Numerous ecdysone receptors are available and can be used with the present invention. These ecdysone receptors include but are not limited to ecdysone receptors from *Drosophila melanogaster* (genbank accession M74078; Koelle *et al.* (1991) *Cell* 67: 59-77), *Manduca sexta* (genbank accession U19812; Fujiwara *et al.* (1995) *Insect Biochem Mol Biol.* 25: 845-856), *Bombyx mori* (genbank accessions L35266 and D43943; Swevers *et al.* (1995) *Insect Biochem Mol Biol* 25: 857-866), *Ostrinia nubilalis* (WO 00/15791A1), *Chironomus tentans* (genbank accession S60739), *Spodoptera exigua* (WO 96/37609), *Locusta migratoria* (genbank accession AF049136; Saleh *et al.* (1998) *Mol Cell Endocrinol* 143: 91-99), *Choristoneura fumiferana* (genbank accession U29531; Kothapalli *et al.* (1995) *Dev. Genet.* 17: 319-330) and *Heliothis virescens* (WO 96/37609). In an additional embodiment of the present invention, novel ecdysone receptor domains are cloned from the insect ecdysone receptors of *Agrotis ipsilon* and *Spodoptera frugiperda.*

A. The Ligand Binding Domain

The ligand binding (E) domain of the receptor polypeptide provides the means by which the 5' regulatory region of the target expression cassette is activated in response to the presence of a chemical ligand. The ecdysone receptor (EcR) from *Drosophila* is one example of a receptor polypeptide where complementary chemical ligands have been identified that bind to the ligand binding domain. The steroid hormone ecdysone triggers coordinate changes in tissue development that results in metamorphosis, and ecdysone has been shown to bind to EcR. (Koelle *et al., Cell* 67: 59-77 (1991)). The plant-produced analog of ecdysone, muristerone, also binds to the ligand binding domain of EcR. Other chemicals, such as the non-steroidal ecdysone agonists RH 5849 (Wing, *Science* 241: 467-469 (1988)), RH-2485 (methoxyfenozide, Dhadialla et al. (1998) Annu Rev Entom 43: 545-569) and RH 5992 (tebufenozide), the latter known as the insecticide MIMIC®, also will act as a chemical ligand for the ligand binding domain of EcR. *See also,* Dhadialla *et al., Annu. Rev. Entomol.* 43:545-569 (1998), incorporated herein by reference, which describes several insecticides with ecdysteroidal and juvenile hormone activity.

The ecdysone receptors from different insects generally have homology among their amino acid sequences. The ligand binding domain of EcR's from closely related insects shows high homology whereas EcR's from less closely related species are more divergent in their amino acid sequences. In one embodiment of the present invention, the ligand binding domain of the chimeric receptor polypeptide is from the ecdysone receptor of an insect selected from the group consisting of *Manduca sexta, Agrotis ipsilon, Ostrinia nubilalis , Spodoptera frugiperda, Locusta migratoria,* and *Chironomus tentans.* In a preferred embodiment, the ligand binding domain is from the ecdysone receptor of *Manduca sexta, Agrotis ipsilon, Ostrinia nubilalis , Spodoptera frugiperda,* or *Chironomus tentans.* These ligand binding domains confer high level activity upon the chimeric receptor polypeptide when the receptor polypeptide is expressed in a cell along with a target expression cassette and exposed to a ligand.

The choice of chemical ligand will depend on which ligand binding domains are present in the receptor polypeptide. Any chemical compound will suffice as long as it is shown to form a complementary binding pair with the chosen ligand binding domain. When a naturally-occuring compound is known to form a complementary binding pair with a particular ligand

binding domain, these known compounds also find use in the present invention. Particularly useful chemicals include but are not limited to insecticides that form a complementary binding pair with the ligand binding domain. Such chemicals include but are not limited to hormones, hormone agonists, and hormone antagonists whose function as insecticides can be acscribed to their binding to native receptor proteins in insects. In addition, chemicals with these hormone or hormone-related properties which are known as insecticides have the additional benefit of already having been examined for agricultural production, making such chemicals "ready-to-use" for field application to crops. Useful chemicals with these properties include but are not limited to RH 5849, RH-2485 (methoxyfenozide), and RH 5992 (tebufenozide),

B. The Hinge Domain

The hinge (D) domain is defined as amino acids of the receptor polypeptide between the DNA binding (C) and ligand binding (E) domains. The activities ascribed to this region are the abilities of the receptor polypeptide to interact with itself in a homodimer or with a second heterologous receptor polypeptide in a heterodimer. Mutations in the hinge region have been shown to alter the ability of the ecdysone receptor to interact with the ultraspiracle receptor and the RXR receptor. In the present invention, the hinge domain is used to modulate the activity of the chimeric receptor polypeptide. In one embodiment of the present invention, the hinge domain of the chimeric receptor polypeptide is from the ecdysone receptor of an insect selected from the group consisting of *Manduca sexta, Agrotis ipsilon, Ostrinia nubilalis , Spodoptera frugiperda, Locusta migratoria,* and *Chironomus tentans*. In a preferred embodiment, the hinge domain is from the ecdysone receptor of *Manduca sexta, Agrotis ipsilon, Ostrinia nubilalis , Spodoptera frugiperda,* or *Locusta migratoria.*

A preferred embodiment of the present invention provides chimeric receptor polypeptides wherein the hinge and ligand binding domains are, with respect to one another, selected from different insect ecdysone receptors. The combination of hinge and ligand binding domains in chimeric receptor polypeptides results in receptor polypeptides with novel activities in response to ligands.

C. The DNA Binding Domain and its Response Elements

The DNA binding (C) domain is a sequence of amino acids having certain functional features that are responsible for binding of the receptor polypeptide to a specific sequence of nucleotides, the response elements, which are present in the 5' regulatory region of the target expression cassette. In one embodiment of the invention, the DNA binding domain is obtained from an insect ecdysone receptor and contains cysteine residues arranged in such a way that, when coordinated by zinc ions, forms the so-called "zinc-finger" motif. The structure of DNA binding domains for the insect ecdysone receptors is highly conserved from one insect species to another, and consequently there is limited variation in the response elements used to form a complementary binding pair (Evans, *Science* 240: 889-895 (1988)). Nevertheless, considerable flexibility can be introduced into the method of controlling gene expression by using these conserved response elements in other ways. In a preferred embodiment of the invention, multiple copies of the appropriate response element are placed in the 5' regulatory region, which allows multiple sites for binding of receptor polypeptide resulting in a greater degree of activation.

Additional flexibility in controlling gene expression by the present invention may be obtained by using DNA binding domains and response elements from other transcriptional activators, which include but are not limited to the LexA or GAL4 proteins. The DNA binding domain from the LexA protein encoded by the lexA gene from E. coli and its complementary binding site (Brent and Ptashne, *Cell* 43: 729-736 (1985), which describes a LexA/GAL4 transcriptional activator) can be utilized. Another useful source is from the GAL4 protein of yeast (Sadowski *et al., Nature* 335: 563-564 (1988), which describes a GAL4-VP16 transcriptional activator). In one preferred embodiment of the invention, a chimeric receptor polypeptide is constructed by fusing the GAL4 DNA binding domain to a moiety containing the hinge and ligand binding domains from *Manduca* EcR, which can control expression of a target expression cassette.

An additional degree of flexibility in controlling gene expression can be obtained by using synthetic DNA binding domains and response elements. Protein engineering experiments have shown that it is possible to rationally alter the DNA binding characteristics of zinc finger domains to bind to a DNA target sequence of choice (Liu *et al., Proc. Natl. Acad. Sci.* 94: 5525-5530 (1997); Desjarlais and Berg, *Proc. Natl. Acad. Sci.* 90: 2256-2260

(1993)). The use of a synthetic zinc finger binding domain allows the chimeric receptor polypeptide to recognize a target sequence of choice. This target sequence may be part of a target cassette transformed into a plant or may be a target sequence in the genome of a plant, to control expression of a native plant gene.

D. The Transactivation Domain

Transactivation (A/B) domains can be defined as amino acid sequences that, when combined with the DNA binding domain in a receptor polypeptide, increase productive transcription initiation by RNA polymerases. (*See generally*, Ptashne, *Nature* 335: 683-689 (1988); Meshi, *Plant Cell Physiol* 36: 1405-1420 (1995)). Different transactivation domains are known to have different degrees of effectiveness in their abilities to increase transcription inititiation. In the present invention, it is desirable to use transactivation domains that have superior transactivating effectiveness in plant cells in order to create a high level of target expression cassette expression in response to the presence of chemical ligand. Transactivation domains that have been shown to be particularly effective in the method of the present invention include but are not limited to VP16 (Triezenberg, *et al., Genes and Dev.* 2(6): 718-729 (1988) - isolated from the herpes simplex virus) and C1 (*Goff et al., Genes and Dev.* 5:298-309 (1991) - isolated from maize), AP1 (isolated from *Arabidopsis*), and Dof1 (isolated from maize). In one preferred embodiment of the present invention, the transactivation domain from VP16 is fused to an EcR moiety for controlling target polypeptide expression in plants. Other transactivation domains may also be effective.

II. Repression of Gene Expression

As described above, the method of the present invention can be used to increase gene expression over a minimal, basal level. One of the outstanding benefits of the present method, however, is that it can also be used for decreasing or inhibiting gene expression, i.e., gene repression. A means of controlling gene expression through repression can be accomplished by using a repression domain in place of the transactivation domain. Repression domains can be defined as amino acid sequences that, when combined with the DNA binding domain in a receptor polypeptide, decrease the productive transcription initiation by RNA polymerases

(Ng, *Trends Biochem. Sci.* 25:121-126 (2000)). Repression domains that can be used with the present invention to decrease expression of a target cassette include but are not limited to the repression domains of AtHD2A (Wu, *Plant J.* 22:19-27(2000)), Oshox1, and Oshox3 (Meijer, *Mol. Gen. Genet.* 263: 12-21 (2000)).

III. Controlling Gene Expression in Transgenic Plants

The invention further comprises a method of controlling plant gene expression comprising transforming a plant with the insect ecdysone receptor cassette encoding a chimeric receptor polypeptide, and at least one target expression cassette. The insect ecdysone receptor cassette is operatively linked with a 5' regulatory region capable of promoting expression in a plant cell, and a 3' terminating region. The target expression cassette comprises a 5' regulatory region operatively linked to a target nucleotide sequence, wherein the 5' regulatory region comprises one or more response elements complementary to the DNA binding domain of the receptor polypeptide. The target expression cassette is activated by the chimeric receptor polypeptide in the presence of one or more chemical ligands and the expression of the target nucleotide sequence is accomplished.

In accordance with a preferred embodiment of the invention, it has been discovered that a chimeric receptor polypeptide comprising the *Manduca* ecdysone receptor hinge and ligand binding domains activates high levels of expression of a target expression cassette in the presence of ligand. Thus a preferred embodiment of the present invention is a method of controlling gene expression in a plant comprising transforming a plant with a target expression cassette and a receptor expression cassette comprising a 5' regulatory region capable of promoting expression in a plant cell, a receptor cassette comprising a DNA binding domain, a hinge domain from the *Manduca sexta* ecdysone receptor, a ligand binding domain from the *Manduca sexta* ecdysone receptor, a transactivation domain, and a 3' terminating region. The target expression cassette is activated by the receptor polypeptide in the presence of one or more chemical ligands and the expression of the target nucleotide sequence is accomplished.

The chimeric receptor polypeptide encoded by the receptor cassette may be expressed in plants when it is operatively linked to a promoter that permits expression in plant tissues and

cells. Appropriate promoters are chosen for the receptor expression cassettes so that expression of the receptor polypeptides may be constitutive, developmentally regulated, tissue specific, cell specific or cell compartment specific. Promoters may also be chosen so that expression of the receptor polypeptides themselves can be chemically-induced in the plant, thereby increasing the level of promoter induction by ligand. By combining promoter elements that confer specific expression with those conferring chemically-induced expression, the receptor polypeptides may be expressed or activated within specific cells or tissues of the plant in response to chemical application. The nucleotide sequence that encodes the receptor polypeptide may be modified for improved expression in plants, improved functionality, or both. Such modifications include, but are not limited to, altering codon usage, insertion of introns or creation of mutations.

Target polypeptides whose expression is activated by the receptor polypeptides in the presence of a chemical ligand are also disclosed. The expression of any coding sequence may be controlled by the present invention, provided that the promoter operatively linked to said coding sequence has been engineered to contain the response element or response elements that are complementary to the DNA binding domain of the receptor polypeptides used. For example, target polypeptides that are useful for controlling plant fertility are activated by the receptor polypeptides in the presence of a chemical ligand.

A. Modification of Coding Sequences and Adjacent Sequences

The transgenic expression in plants of genes derived from heterologous sources may involve the modification of those genes to achieve and optimize their expression in plants. In particular, bacterial ORFs which encode separate enzymes but which are encoded by the same transcript in the native microbe are best expressed in plants on separate transcripts. To achieve this, each microbial ORF is isolated individually and cloned within a cassette which provides a plant promoter sequence at the 5' end of the ORF and a plant transcriptional terminator at the 3' end of the ORF. The isolated ORF sequence preferably includes the initiating ATG codon and the terminating STOP codon but may include additional sequence beyond the initiating ATG and the STOP codon. In addition, the ORF may be truncated, but still retain the required activity; for particularly long ORFs, truncated versions which retain activity may be preferable for expression in transgenic organisms. By "plant promoter" and

"plant transcriptional terminator" it is intended to mean promoters and transcriptional terminators which operate within plant cells. This includes promoters and transcription terminators which may be derived from non-plant sources such as viruses (an example is the Cauliflower Mosaic Virus).

In some cases, modification to the ORF coding sequences and adjacent sequence is not required. It is sufficient to isolate a fragment containing the ORF of interest and to insert it downstream of a plant promoter. For example, Gaffney *et al.* (*Science* 261: 754-756 (1993)) have expressed the *Pseudomonas nahG* gene in transgenic plants under the control of the CaMV 35S promoter and the CaMV *tml* terminator successfully without modification of the coding sequence and with nucleotides of the *Pseudomonas* gene upstream of the ATG still attached, and nucleotides downstream of the STOP codon still attached to the *nahG* ORF. Preferably as little adjacent microbial sequence should be left attached upstream of the ATG and downstream of the STOP codon. In practice, such construction may depend on the availability of restriction sites.

In other cases, the expression of genes derived from microbial sources may provide problems in expression. These problems have been well characterized in the art and are particularly common with genes derived from certain sources such as *Bacillus*. These problems may apply to the nucleotide sequence of this invention and the modification of these genes can be undertaken using techniques now well known in the art. The following problems may be encountered:

1. Codon Usage.

The preferred codon usage in plants differs from the preferred codon usage in certain microorganisms. Comparison of the usage of codons within a cloned microbial ORF to usage in plant genes (and in particular genes from the target plant) will enable an identification of the codons within the ORF which should preferably be changed. Typically plant evolution has tended towards a strong preference of the nucleotides C and G in the third base position of monocotyledons, whereas dicotyledons often use the nucleotides A or T at this position. By modifying a gene to incorporate preferred codon usage for a particular target transgenic species, many of the problems described below for GC/AT content and illegitimate splicing will be overcome.

2. GC/AT Content.

Plant genes typically have a GC content of more than 35%. ORF sequences which are rich in A and T nucleotides can cause several problems in plants. Firstly, motifs of ATTTA are believed to cause destabilization of messages and are found at the 3' end of many short-lived mRNAs. Secondly, the occurrence of polyadenylation signals such as AATAAA at inappropriate positions within the message is believed to cause premature truncation of transcription. In addition, monocotyledons may recognize AT-rich sequences as splice sites (see below).

3. Sequences Adjacent to the Initiating Methionine.

Plants differ from microorganisms in that their messages do not possess a defined ribosome binding site. Rather, it is believed that ribosomes attach to the 5' end of the message and scan for the first available ATG at which to start translation. Nevertheless, it is believed that there is a preference for certain nucleotides adjacent to the ATG and that expression of microbial genes can be enhanced by the inclusion of a eukaryotic consensus translation initiator at the ATG. Clontech (1993/1994 catalog, page 210, incorporated herein by reference) have suggested one sequence as a consensus translation initiator for the expression of the *E. coli uidA* gene in plants. Further, Joshi (*N.A.R.* 15: 6643-6653 (1987), incorporated herein by reference) has compared many plant sequences adjacent to the ATG and suggests another consensus sequence. In situations where difficulties are encountered in the expression of microbial ORFs in plants, inclusion of one of these sequences at the initiating ATG may improve translation. In such cases the last three nucleotides of the consensus may not be appropriate for inclusion in the modified sequence due to their modification of the second AA residue. Preferred sequences adjacent to the initiating methionine may differ between different plant species. A survey of 14 maize genes located in the GenBank database provided the following results:

Position Before the Initiating ATG in 14 Maize Genes:

	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1
C	3	8	4	6	2	5	6	0	10	7
T	3	0	3	4	3	2	1	1	1	0
A	2	3	1	4	3	2	3	7	2	3
G	6	3	6	0	6	5	4	6	1	5

This analysis can be done for the desired plant species into which the nucleotide sequence is being incorporated, and the sequence adjacent to the ATG modified to incorporate the preferred nucleotides.

4. Removal of Illegitimate Splice Sites.

Genes cloned from non-plant sources and not optimized for expression in plants may also contain motifs which may be recognized in plants as 5' or 3' splice sites, and be cleaved, thus generating truncated or deleted messages. These sites can be removed using the techniques well known in the art.

Techniques for the modification of coding sequences and adjacent sequences are well known in the art. In cases where the initial expression of a microbial ORF is low and it is deemed appropriate to make alterations to the sequence as described above, then the construction of synthetic genes can be accomplished according to methods well known in the art. These are, for example, described in the published patent disclosures EP 0 385 962 (to Monsanto), EP 0 359 472 (to Lubrizol) and WO 93/07278 (to Ciba-Geigy), all of which are incorporated herein by reference. In most cases it is preferable to assay the expression of gene constructions using transient assay protocols (which are well known in the art) prior to their transfer to transgenic plants.

B. Construction of Plant Expression Cassettes

Coding sequences intended for expression in transgenic plants are first assembled in expression cassettes behind a suitable promoter expressible in plants. The expression cassettes may also comprise any further sequences required or selected for the expression of the transgene. Such sequences include, but are not restricted to, transcription terminators,

extraneous sequences to enhance expression such as introns, vital sequences, and sequences intended for the targeting of the gene product to specific organelles and cell compartments. These expression cassettes can then be easily transferred to the plant transformation vectors described below. The following is a description of various components of typical expression cassettes.

1. Promoters

The selection of the promoter used in expression cassettes will determine the spatial and temporal expression pattern of the transgene in the transgenic plant. Selected promoters will express transgenes in specific cell types (such as leaf epidermal cells, mesophyll cells, root cortex cells) or in specific tissues or organs (roots, leaves or flowers, for example) and the selection will reflect the desired location of accumulation of the gene product. Alternatively, the selected promoter may drive expression of the gene under various inducing conditions. Promoters vary in their strength, i.e., ability to promote transcription. Depending upon the host cell system utilized, any one of a number of suitable promoters can be used, including the gene's native promoter. The following are non-limiting examples of promoters that may be used in expression cassettes.

a. Constitutive Expression, the Ubiquitin Promoter:

Ubiquitin is a gene product known to accumulate in many cell types and its promoter has been cloned from several species for use in transgenic plants (*e.g.* sunflower - Binet *et al.* Plant Science 79: 87-94 (1991); maize - Christensen *et al.* Plant Molec. Biol. 12: 619-632 (1989); and *Arabidopsis* - Callis *et al., J. Biol. Chem.* 265:12486-12493 (1990) and Norris *et al., Plant Mol. Biol.* 21:895-906 (1993)). The maize ubiquitin promoter has been developed in transgenic monocot systems and its sequence and vectors constructed for monocot transformation are disclosed in the patent publication EP 0 342 926 (to Lubrizol) which is herein incorporated by reference. Taylor *et al.* (Plant Cell Rep. 12: 491-495 (1993)) describe a vector (pAHC25) that comprises the maize ubiquitin promoter and first intron and its high activity in cell suspensions of numerous monocotyledons when introduced via microprojectile bombardment. The *Arabidopsis* ubiquitin promoter is ideal for use with the nucleotide sequences of the present invention. The ubiquitin promoter is suitable for gene expression in

transgenic plants, both monocotyledons and dicotyledons. Suitable vectors are derivatives of pAHC25 or any of the transformation vectors described in this application, modified by the introduction of the appropriate ubiquitin promoter and/or intron sequences.

b. Constitutive Expression, the CaMV 35S Promoter:

Construction of the plasmid pCGN1761 is described in the published patent application EP 0 392 225 (Example 23), which is hereby incorporated by reference. pCGN1761 contains the "double" CaMV 35S promoter and the *tml* transcriptional terminator with a unique *EcoRI* site between the promoter and the terminator and has a pUC-type backbone. A derivative of pCGN1761 is constructed which has a modified polylinker which includes *NotI* and *XhoI* sites in addition to the existing *EcoRI* site. This derivative is designated pCGN1761ENX. pCGN1761ENX is useful for the cloning of cDNA sequences or coding sequences (including microbial ORF sequences) within its polylinker for the purpose of their expression under the control of the 35S promoter in transgenic plants. The entire 35S promoter-coding sequence-*tml* terminator cassette of such a construction can be excised by *HindIII, SphI, SalI,* and *XbaI* sites 5' to the promoter and *XbaI, BamHI* and *BglI* sites 3' to the terminator for transfer to transformation vectors such as those described below. Furthermore, the double 35S promoter fragment can be removed by 5' excision with *HindIII, SphI, SalI, XbaI*, or *PstI*, and 3' excision with any of the polylinker restriction sites (*EcoRI, NotI* or *XhoI*) for replacement with another promoter. If desired, modifications around the cloning sites can be made by the introduction of sequences that may enhance translation. This is particularly useful when overexpression is desired. For example, pCGN1761ENX may be modified by optimization of the translational initiation site as described in Example 37 of U.S. Patent No. 5,639,949, incorporated herein by reference.

c. Constitutive Expression, the Actin Promoter:

Several isoforms of actin are known to be expressed in most cell types and consequently the actin promoter is a good choice for a constitutive promoter. In particular, the promoter from the rice *ActI* gene has been cloned and characterized (McElroy *et al.* Plant Cell 2: 163-171 (1990)). A 1.3kb fragment of the promoter was found to contain all the regulatory elements required for expression in rice protoplasts. Furthermore, numerous expression

vectors based on the *ActI* promoter have been constructed specifically for use in monocotyledons (McElroy *et al.* Mol. Gen. Genet. 231: 150-160 (1991)). These incorporate the *ActI*-intron 1, *AdhI* 5' flanking sequence and *AdhI*-intron 1 (from the maize alcohol dehydrogenase gene) and sequence from the CaMV 35S promoter. Vectors showing highest expression were fusions of 35S and *ActI* intron or the *ActI* 5' flanking sequence and the *ActI* intron. Optimization of sequences around the initiating ATG (of the GUS reporter gene) also enhanced expression. The promoter expression cassettes described by McElroy *et al.* (Mol. Gen. Genet. 231: 150-160 (1991)) can be easily modified for gene expression and are particularly suitable for use in monocotyledonous hosts. For example, promoter-containing fragments is removed from the McElroy constructions and used to replace the double 35S promoter in pCGN1761ENX, which is then available for the insertion of specific gene sequences. The fusion genes thus constructed can then be transferred to appropriate transformation vectors. In a separate report, the rice *ActI* promoter with its first intron has also been found to direct high expression in cultured barley cells (Chibbar *et al.* Plant Cell Rep. 12: 506-509 (1993)).

d. Inducible Expression, PR-1 Promoters:

The double 35S promoter in pCGN1761ENX may be replaced with any other promoter of choice that will result in suitably high expression levels. By way of example, one of the chemically regulatable promoters described in U.S. Patent No. 5,614,395, such as the tobacco PR-1a promoter, may replace the double 35S promoter. Alternately, the *Arabidopsis* PR-1 promoter described in Lebel *et al., Plant J.* 16:223-233 (1998) may be used. The promoter of choice is preferably excised from its source by restriction enzymes, but can alternatively be PCR-amplified using primers that carry appropriate terminal restriction sites. Should PCR-amplification be undertaken, then the promoter should be re-sequenced to check for amplification errors after the cloning of the amplified promoter in the target vector. The chemically/pathogen regulatable tobacco PR-1a promoter is cleaved from plasmid pCIB1004 (for construction, see example 21 of EP 0 332 104, which is hereby incorporated by reference) and transferred to plasmid pCGN1761ENX (Uknes *et al., Plant Cell* 4: 645-656 (1992)). pCIB1004 is cleaved with *NcoI* and the resultant 3' overhang of the linearized fragment is rendered blunt by treatment with T4 DNA polymerase. The fragment is then cleaved with

HindIII and the resultant PR-1a promoter-containing fragment is gel purified and cloned into pCGN1761ENX from which the double 35S promoter has been removed. This is done by cleavage with *XhoI* and blunting with T4 polymerase, followed by cleavage with *HindIII* and isolation of the larger vector-terminator containing fragment into which the pCIB1004 promoter fragment is cloned. This generates a pCGN1761ENX derivative with the PR-1a promoter and the *tml* terminator and an intervening polylinker with unique *EcoRI* and *NotI* sites. The selected coding sequence can be inserted into this vector, and the fusion products (*i.e.* promoter-gene-terminator) can subsequently be transferred to any selected transformation vector, including those described *infra*. Various chemical regulators may be employed to induce expression of the selected coding sequence in the plants transformed according to the present invention, including the benzothiadiazole, isonicotinic acid, and salicylic acid compounds disclosed in U.S. Patent Nos. 5,523,311 and 5,614,395.

e. Inducible Expression, an Ethanol-Inducible Promoter:

A promoter inducible by certain alcohols or ketones, such as ethanol, may also be used to confer inducible expression of a coding sequence of the present invention. Such a promoter is for example the *alcA* gene promoter from *Aspergillus nidulans* (Caddick et al. (1998) *Nat. Biotechnol* 16:177-180). In *A. nidulans*, the *alcA* gene encodes alcohol dehydrogenase I, the expression of which is regulated by the AlcR transcription factors in presence of the chemical inducer. For the purposes of the present invention, the CAT coding sequences in plasmid palcA:CAT comprising a *alcA* gene promoter sequence fused to a minimal 35S promoter (Caddick et al. (1998) *Nat. Biotechnol* 16:177-180) are replaced by a coding sequence of the present invention to form an expression cassette having the coding sequence under the control of the *alcA* gene promoter. This is carried out using methods well known in the art.

f. Inducible Expression, a Glucocorticoid-Inducible Promoter:

Induction of expression of a nucleic acid sequence of the present invention using systems based on steroid hormones is also contemplated. For example, a glucocorticoid-mediated induction system is used (Aoyama and Chua (1997) *The Plant Journal* 11: 605-612) and gene expression is induced by application of a glucocorticoid, for example a synthetic glucocorticoid, preferably dexamethasone, preferably at a concentration ranging from 0.1mM

to 1mM, more preferably from 10mM to 100mM. For the purposes of the present invention, the luciferase gene sequences are replaced by a nucleic acid sequence of the invention to form an expression cassette having a nucleic acid sequence of the invention under the control of six copies of the GAL4 upstream activating sequences fused to the 35S minimal promoter. This is carried out using methods well known in the art. The trans-acting factor comprises the GAL4 DNA-binding domain (Keegan et al. (1986) *Science* 231: 699-704) fused to the transactivating domain of the herpes viral protein VP16 (Triezenberg et al. (1988) *Genes Devel.* 2: 718-729) fused to the hormone-binding domain of the rat glucocorticoid receptor (Picard et al. (1988) *Cell* 54: 1073-1080). The expression of the fusion protein is controlled by any promoter suitable for expression in plants known in the art or described here. This expression cassette is also comprised in the plant comprising a nucleic acid sequence of the invention fused to the 6xGAL4/minimal promoter. Thus, tissue- or organ-specificity of the fusion protein is achieved leading to inducible tissue- or organ-specificity of the insecticidal toxin.

g. Root Specific Expression:

Another pattern of gene expression is root expression. A suitable root promoter is the promoter of the maize metallothionein-like (MTL) gene described by de Framond (FEBS 290: 103-106 (1991)) and also in U.S. Patent No. 5,466,785, incorporated herein by reference. This "MTL" promoter is transferred to a suitable vector such as pCGN1761ENX for the insertion of a selected gene and subsequent transfer of the entire promoter-gene-terminator cassette to a transformation vector of interest.

h. Wound-Inducible Promoters:

Wound-inducible promoters may also be suitable for gene expression. Numerous such promoters have been described (*e.g.* Xu *et al.* Plant Molec. Biol. 22: 573-588 (1993), Logemann *et al.* Plant Cell 1: 151-158 (1989), Rohrmeier & Lehle, Plant Molec. Biol. 22: 783-792 (1993), Firek *et al.* Plant Molec. Biol. 22: 129-142 (1993), Warner *et al.* Plant J. 3: 191-201 (1993)) and all are suitable for use with the instant invention. Logemann *et al.* describe the 5' upstream sequences of the dicotyledonous potato *wunI* gene. Xu *et al.* show that a wound-inducible promoter from the dicotyledon potato (*pin2*) is active in the monocotyledon rice. Further, Rohrmeier & Lehle describe the cloning of the maize *WipI*

cDNA which is wound induced and which can be used to isolate the cognate promoter using standard techniques. Similar, Firek *et al.* and Warner *et al.* have described a wound-induced gene from the monocotyledon *Asparagus officinalis,* which is expressed at local wound and pathogen invasion sites. Using cloning techniques well known in the art, these promoters can be transferred to suitable vectors, fused to the genes pertaining to this invention, and used to express these genes at the sites of plant wounding.

i. Pith-Preferred Expression:

Patent Application WO 93/07278, which is herein incorporated by reference, describes the isolation of the maize *trpA* gene, which is preferentially expressed in pith cells. The gene sequence and promoter extending up to -1726 bp from the start of transcription are presented. Using standard molecular biological techniques, this promoter, or parts thereof, can be transferred to a vector such as pCGN1761 where it can replace the 35S promoter and be used to drive the expression of a foreign gene in a pith-preferred manner. In fact, fragments containing the pith-preferred promoter or parts thereof can be transferred to any vector and modified for utility in transgenic plants.

j. Leaf-Specific Expression:

A maize gene encoding phosphoenol carboxylase (PEPC) has been described by Hudspeth & Grula (Plant Molec Biol 12: 579-589 (1989)). Using standard molecular biological techniques the promoter for this gene can be used to drive the expression of any gene in a leaf-specific manner in transgenic plants.

k. Pollen-Specific Expression:

WO 93/07278 describes the isolation of the maize calcium-dependent protein kinase (CDPK) gene which is expressed in pollen cells. The gene sequence and promoter extend up to 1400 bp from the start of transcription. Using standard molecular biological techniques, this promoter or parts thereof, can be transferred to a vector such as pCGN1761 where it can replace the 35S promoter and be used to drive the expression of a nucleic acid sequence of the invention in a pollen-specific manner.

2. Transcriptional Terminators

A variety of transcriptional terminators are available for use in expression cassettes. These are responsible for the termination of transcription beyond the transgene and correct mRNA polyadenylation. Appropriate transcriptional terminators are those that are known to function in plants and include the CaMV 35S terminator, the *tml* terminator, the nopaline synthase terminator and the pea *rbcS* E9 terminator. These can be used in both monocotyledons and dicotyledons. In addition, a gene's native transcription terminator may be used.

3. Sequences for the Enhancement or Regulation of Expression

Numerous sequences have been found to enhance gene expression from within the transcriptional unit and these sequences can be used in conjunction with the genes of this invention to increase their expression in transgenic plants.

Various intron sequences have been shown to enhance expression, particularly in monocotyledonous cells. For example, the introns of the maize *AdhI* gene have been found to significantly enhance the expression of the wild-type gene under its cognate promoter when introduced into maize cells. Intron 1 was found to be particularly effective and enhanced expression in fusion constructs with the chloramphenicol acetyltransferase gene (Callis *et al.*, *Genes Develop.* 1: 1183-1200 (1987)). In the same experimental system, the intron from the maize *bronze1* gene had a similar effect in enhancing expression. Intron sequences have been routinely incorporated into plant transformation vectors, typically within the non-translated leader.

A number of non-translated leader sequences derived from viruses are also known to enhance expression, and these are particularly effective in dicotyledonous cells. Specifically, leader sequences from Tobacco Mosaic Virus (TMV, the "W-sequence"), Maize Chlorotic Mottle Virus (MCMV), and Alfalfa Mosaic Virus (AMV) have been shown to be effective in enhancing expression (*e.g.* Gallie *et al. Nucl. Acids Res.* 15: 8693-8711 (1987); Skuzeski *et al. Plant Molec. Biol.* 15: 65-79 (1990)). Other leader sequences known in the art include but are not limited to: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein, O., Fuerst, T. R., and Moss, B. *PNAS USA* 86:6126-6130 (1989)); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison et al.,

1986); MDMV leader (Maize Dwarf Mosaic Virus); *Virology* 154:9-20); human immunoglobulin heavy-chain binding protein (BiP) leader, (Macejak, D. G., and Sarnow, P., *Nature* 353: 90-94 (1991); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4), (Jobling, S. A., and Gehrke, L., *Nature* 325:622-625 (1987); tobacco mosaic virus leader (TMV), (Gallie, D. R. et al., *Molecular Biology of RNA*, pages 237-256 (1989); and Maize Chlorotic Mottle Virus leader (MCMV) (Lommel, S. A. et al., *Virology* 81:382-385 (1991). See also, Della-Cioppa et al., *Plant Physiology* 84:965-968 (1987).

In addition to incorporating one or more of the aforementioned elements into the 5' regulatory region of a target expression cassette of the invention, other elements peculiar to the target expression cassette may also be incorporated. Such elements include but are not limited to a minimal promoter. By minimal promoter it is intended that the basal promoter elements are inactive or nearly so without upstream activation. Such a promoter has low background activity in plants when there is no transactivator present or when enhancer or response element binding sites are absent. One minimal promoter that is particularly useful for target genes in plants is the Bz1 minimal promoter, which is obtained from the bronze1 gene of maize. The Bz1 core promoter is obtained from the "myc" mutant Bz1-luciferase construct pBz1LucR98 via cleavage at the NheI site located at -53 to -58. Roth *et al., Plant Cell* 3: 317 (1991). The derived Bz1 core promoter fragment thus extends from -53 to +227 and includes the Bz1 intron-1 in the 5' untranslated region. Also useful for the invention is a minimal promoter created by use of a synthetic TATA element. The TATA element allows recognition of the promoter by RNA polymerase factors and confers a basal level of gene expression in the absence of activation (*see generally*, Mukumoto (1993) *Plant Mol Biol* 23: 995-1003; Green (2000) *Trends Biochem Sci* 25: 59-63)

4. Targeting of the Gene Product Within the Cell

Various mechanisms for targeting gene products are known to exist in plants and the sequences controlling the functioning of these mechanisms have been characterized in some detail. For example, the targeting of gene products to the chloroplast is controlled by a signal sequence found at the amino terminal end of various proteins which is cleaved during chloroplast import to yield the mature protein (*e.g.* Comai *et al.* J. Biol. Chem. 263: 15104-15109 (1988)). These signal sequences can be fused to heterologous gene products to effect

the import of heterologous products into the chloroplast (van den Broeck, et al. Nature 313: 358-363 (1985)). DNA encoding for appropriate signal sequences can be isolated from the 5' end of the cDNAs encoding the RUBISCO protein, the CAB protein, the EPSP synthase enzyme, the GS2 protein and many other proteins which are known to be chloroplast localized. *See also*, the section entitled "Expression With Chloroplast Targeting" in Example 37 of U.S. Patent No. 5,639,949.

Other gene products are localized to other organelles such as the mitochondrion and the peroxisome (*e.g.* Unger *et al.* Plant Molec. Biol. 13: 411-418 (1989)). The cDNAs encoding these products can also be manipulated to effect the targeting of heterologous gene products to these organelles. Examples of such sequences are the nuclear-encoded ATPases and specific aspartate amino transferase isoforms for mitochondria. Targeting cellular protein bodies has been described by Rogers *et al.* (Proc. Natl. Acad. Sci. USA 82: 6512-6516 (1985)).

In addition, sequences have been characterized which cause the targeting of gene products to other cell compartments. Amino terminal sequences are responsible for targeting to the ER, the apoplast, and extracellular secretion from aleurone cells (Koehler & Ho, Plant Cell 2: 769-783 (1990)). Additionally, amino terminal sequences in conjunction with carboxy terminal sequences are responsible for vacuolar targeting of gene products (Shinshi *et al.* Plant Molec. Biol. 14: 357-368 (1990)).

By the fusion of the appropriate targeting sequences described above to transgene sequences of interest it is possible to direct the transgene product to any organelle or cell compartment. For chloroplast targeting, for example, the chloroplast signal sequence from the RUBISCO gene, the CAB gene, the EPSP synthase gene, or the GS2 gene is fused in frame to the amino terminal ATG of the transgene. The signal sequence selected should include the known cleavage site, and the fusion constructed should take into account any amino acids after the cleavage site which are required for cleavage. In some cases this requirement may be fulfilled by the addition of a small number of amino acids between the cleavage site and the transgene ATG or, alternatively, replacement of some amino acids within the transgene sequence. Fusions constructed for chloroplast import can be tested for efficacy of chloroplast uptake by *in vitro* translation of *in vitro* transcribed constructions followed by *in vitro* chloroplast uptake using techniques described by Bartlett *et al.* In: Edelmann *et al.* (Eds.) Methods in Chloroplast Molecular Biology, Elsevier pp 1081-1091 (1982) and Wasmann *et*

al. Mol. Gen. Genet. 205: 446-453 (1986). These construction techniques are well known in the art and are equally applicable to mitochondria and peroxisomes.

The above-described mechanisms for cellular targeting can be utilized not only in conjunction with their cognate promoters, but also in conjunction with heterologous promoters so as to effect a specific cell-targeting goal under the transcriptional regulation of a promoter that has an expression pattern different to that of the promoter from which the targeting signal derives.

C. Construction of Plant Transformation Vectors

Numerous transformation vectors available for plant transformation are known to those of ordinary skill in the plant transformation arts, and the genes pertinent to this invention can be used in conjunction with any such vectors. The selection of vector will depend upon the preferred transformation technique and the target species for transformation. For certain target species, different antibiotic or herbicide selection markers may be preferred. Selection markers used routinely in transformation include the *nptII* gene, which confers resistance to kanamycin and related antibiotics (Messing & Vierra. Gene 19: 259-268 (1982); Bevan et al., Nature 304:184-187 (1983)), the *bar* gene, which confers resistance to the herbicide phosphinothricin (White et al., Nucl. Acids Res 18: 1062 (1990), Spencer et al. Theor. Appl. Genet 79: 625-631 (1990)), the *hph* gene, which confers resistance to the antibiotic hygromycin (Blochinger & Diggelmann, Mol Cell Biol 4: 2929-2931), and the *dhfr* gene, which confers resistance to methatrexate (Bourouis et al., EMBO J. 2(7): 1099-1104 (1983)), the EPSPS gene, which confers resistance to glyphosate (U.S. Patent Nos. 4,940,935 and 5,188,642), and the mannose-6-phosphate isomerase gene, which provides the ability to metabolize mannose (U.S. Patent Nos. 5,767,378 and 5,994,629).

1. Vectors Suitable for *Agrobacterium* Transformation

Many vectors are available for transformation using *Agrobacterium tumefaciens*. These typically carry at least one T-DNA border sequence and include vectors such as pBIN19 (Bevan, Nucl. Acids Res. (1984)). Below, the construction of two typical vectors suitable for *Agrobacterium* transformation is described.

a. pCIB200 and pCIB2001:

The binary vectors pCIB200 and pCIB2001 are used for the construction of recombinant vectors for use with *Agrobacterium* and are constructed in the following manner. pTJS75kan is created by *NarI* digestion of pTJS75 (Schmidhauser & Helinski, J. Bacteriol. 164: 446-455 (1985)) allowing excision of the tetracycline-resistance gene, followed by insertion of an *AccI* fragment from pUC4K carrying an NPTII (Messing & Vierra, Gene 19: 259-268 (1982): Bevan et al., Nature 304: 184-187 (1983): McBride et al., Plant Molecular Biology 14: 266-276 (1990)). *XhoI* linkers are ligated to the *EcoRV* fragment of PCIB7 which contains the left and right T-DNA borders, a plant selectable *nos/nptII* chimeric gene and the pUC polylinker (Rothstein et al., Gene 53: 153-161 (1987)), and the *XhoI*-digested fragment are cloned into *SalI*-digested pTJS75kan to create pCIB200 (see also EP 0 332 104, example 19). pCIB200 contains the following unique polylinker restriction sites: *EcoRI, SstI, KpnI, BglII, XbaI,* and *SalI.* pCIB2001 is a derivative of pCIB200 created by the insertion into the polylinker of additional restriction sites. Unique restriction sites in the polylinker of pCIB2001 are *EcoRI, SstI, KpnI, BglII, XbaI, SalI, MluI, BclI, AvrII, ApaI, HpaI,* and *StuI.* pCIB2001, in addition to containing these unique restriction sites also has plant and bacterial kanamycin selection, left and right T-DNA borders for *Agrobacterium*-mediated transformation, the RK2-derived *trfA* function for mobilization between *E. coli* and other hosts, and the *OriT* and *OriV* functions also from RK2. The pCIB2001 polylinker is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

b. pCIB10 and Hygromycin Selection Derivatives thereof:

The binary vector pCIB10 contains a gene encoding kanamycin resistance for selection in plants and T-DNA right and left border sequences and incorporates sequences from the wide host-range plasmid pRK252 allowing it to replicate in both *E. coli* and *Agrobacterium.* Its construction is described by Rothstein *et al.* (Gene 53: 153-161 (1987)). Various derivatives of pCIB10 are constructed which incorporate the gene for hygromycin B phosphotransferase described by Gritz *et al.* (Gene 25: 179-188 (1983)). These derivatives enable selection of transgenic plant cells on hygromycin only (pCIB743), or hygromycin and kanamycin (pCIB715, pCIB717).

2. Vectors Suitable for non-*Agrobacterium* Transformation

Transformation without the use of *Agrobacterium tumefaciens* circumvents the requirement for T-DNA sequences in the chosen transformation vector and consequently vectors lacking these sequences can be utilized in addition to vectors such as the ones described above which contain T-DNA sequences. Transformation techniques that do not rely on *Agrobacterium* include transformation via particle bombardment, protoplast uptake (*e.g.* PEG and electroporation) and microinjection. The choice of vector depends largely on the preferred selection for the species being transformed. Below, the construction of typical vectors suitable for non-*Agrobacterium* transformation is described.

a. pCIB3064:

pCIB3064 is a pUC-derived vector suitable for direct gene transfer techniques in combination with selection by the herbicide basta (or phosphinothricin). The plasmid pCIB246 comprises the CaMV 35S promoter in operational fusion to the *E. coli* GUS gene and the CaMV 35S transcriptional terminator and is described in the PCT published application WO 93/07278. The 35S promoter of this vector contains two ATG sequences 5' of the start site. These sites are mutated using standard PCR techniques in such a way as to remove the ATGs and generate the restriction sites *SspI* and *PvuII*. The new restriction sites are 96 and 37 bp away from the unique *SalI* site and 101 and 42 bp away from the actual start site. The resultant derivative of pCIB246 is designated pCIB3025. The GUS gene is then excised from pCIB3025 by digestion with *SalI* and *SacI*, the termini rendered blunt and religated to generate plasmid pCIB3060. The plasmid pJIT82 is obtained from the John Innes Centre, Norwich and the a 400 bp *SmaI* fragment containing the *bar* gene from *Streptomyces viridochromogenes* is excised and inserted into the *HpaI* site of pCIB3060 (Thompson *et al.* EMBO J 6: 2519-2523 (1987)). This generated pCIB3064, which comprises the *bar* gene under the control of the CaMV 35S promoter and terminator for herbicide selection, a gene for ampicillin resistance (for selection in *E. coli*) and a polylinker with the unique sites *SphI, PstI, HindIII,* and *BamHI*. This vector is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

b. pSOG19 and pSOG35:

pSOG35 is a transformation vector that utilizes the *E. coli* gene dihydrofolate reductase (DFR) as a selectable marker conferring resistance to methotrexate. PCR is used to amplify the 35S promoter (-800 bp), intron 6 from the maize Adh1 gene (-550 bp) and 18 bp of the GUS untranslated leader sequence from pSOG10. A 250-bp fragment encoding the *E. coli* dihydrofolate reductase type II gene is also amplified by PCR and these two PCR fragments are assembled with a *SacI-PstI* fragment from pB1221 (Clontech) which comprises the pUC19 vector backbone and the nopaline synthase terminator. Assembly of these fragments generates pSOG19 which contains the 35S promoter in fusion with the intron 6 sequence, the GUS leader, the DHFR gene and the nopaline synthase terminator. Replacement of the GUS leader in pSOG19 with the leader sequence from Maize Chlorotic Mottle Virus (MCMV) generates the vector pSOG35. pSOG19 and pSOG35 carry the pUC gene for ampicillin resistance and have *HindIII, SphI, PstI* and *EcoRI* sites available for the cloning of foreign substances.

3. Vector Suitable for Chloroplast Transformation

For expression of a nucleotide sequence of the present invention in plant plastids, plastid transformation vector pPH143 (WO 97/32011, example 36) is used. The nucleotide sequence is inserted into pPH143 thereby replacing the PROTOX coding sequence. This vector is then used for plastid transformation and selection of transformants for spectinomycin resistance. Alternatively, the nucleotide sequence is inserted in pPH143 so that it replaces the aadH gene. In this case, transformants are selected for resistance to PROTOX inhibitors.

D. Transformation

Once a nucleic acid sequence of the invention has been cloned into an expression system, it is transformed into a plant cell. The receptor and target expression cassettes of the present invention can be introduced into the plant cell in a number of art-recognized ways. Methods for regeneration of plants are also well known in the art. For example, Ti plasmid vectors have been utilized for the delivery of foreign DNA, as well as direct DNA uptake, liposomes, electroporation, microinjection, and microprojectiles. In addition, bacteria from the genus *Agrobacterium* can be utilized to transform plant cells. Below are descriptions of

representative techniques for transforming both dicotyledonous and monocotyledonous plants, as well as a representative plastid transformation technique.

1. Transformation of Dicotyledons

Transformation techniques for dicotyledons are well known in the art and include *Agrobacterium*-based techniques and techniques that do not require *Agrobacterium*. Non-*Agrobacterium* techniques involve the uptake of exogenous genetic material directly by protoplasts or cells. This can be accomplished by PEG or electroporation mediated uptake, particle bombardment-mediated delivery, or microinjection. Examples of these techniques are described by Paszkowski *et al.*, EMBO J 3: 2717-2722 (1984), Potrykus *et al.*, Mol. Gen. Genet. 199: 169-177 (1985), Reich *et al.*, Biotechnology 4: 1001-1004 (1986), and Klein *et al.*, Nature 327: 70-73 (1987). In each case the transformed cells are regenerated to whole plants using standard techniques known in the art.

Agrobacterium-mediated transformation is a preferred technique for transformation of dicotyledons because of its high efficiency of transformation and its broad utility with many different species. *Agrobacterium* transformation typically involves the transfer of the binary vector carrying the foreign DNA of interest (*e.g.* pCIB200 or pCIB2001) to an appropriate *Agrobacterium* strain which may depend of the complement of *vir* genes carried by the host *Agrobacterium* strain either on a co-resident Ti plasmid or chromosomally (*e.g.* strain CIB542 for pCIB200 and pCIB2001 (Uknes *et al.* Plant Cell 5: 159-169 (1993)). The transfer of the recombinant binary vector to *Agrobacterium* is accomplished by a triparental mating procedure using *E. coli* carrying the recombinant binary vector, a helper *E. coli* strain which carries a plasmid such as pRK2013 and which is able to mobilize the recombinant binary vector to the target *Agrobacterium* strain. Alternatively, the recombinant binary vector can be transferred to *Agrobacterium* by DNA transformation (Höfgen & Willmitzer, Nucl. Acids Res. 16: 9877 (1988)).

Transformation of the target plant species by recombinant *Agrobacterium* usually involves co-cultivation of the *Agrobacterium* with explants from the plant and follows protocols well known in the art. Transformed tissue is regenerated on selectable medium carrying the antibiotic or herbicide resistance marker present between the binary plasmid T-DNA borders.

Another approach to transforming plant cells with a gene involves propelling inert or biologically active particles at plant tissues and cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792 all to Sanford et al. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and afford incorporation within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the desired gene. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried yeast cells, dried bacterium or a bacteriophage, each containing DNA sought to be introduced) can also be propelled into plant cell tissue.

2. Transformation of Monocotyledons

Transformation of most monocotyledon species has now also become routine. Preferred techniques include direct gene transfer into protoplasts using PEG or electroporation techniques, and particle bombardment into callus tissue. Transformations can be undertaken with a single DNA species or multiple DNA species (*i.e.* co-transformation) and both these techniques are suitable for use with this invention. Co-transformation may have the advantage of avoiding complete vector construction and of generating transgenic plants with unlinked loci for the gene of interest and the selectable marker, enabling the removal of the selectable marker in subsequent generations, should this be regarded desirable. However, a disadvantage of the use of co-transformation is the less than 100% frequency with which separate DNA species are integrated into the genome (Schocher *et al.* Biotechnology 4: 1093-1096 (1986)).

Patent Applications EP 0 292 435, EP 0 392 225, and WO 93/07278 describe techniques for the preparation of callus and protoplasts from an elite inbred line of maize, transformation of protoplasts using PEG or electroporation, and the regeneration of maize plants from transformed protoplasts. Gordon-Kamm *et al.* (Plant Cell 2: 603-618 (1990)) and Fromm *et al.* (Biotechnology 8: 833-839 (1990)) have published techniques for transformation of A188-derived maize line using particle bombardment. Furthermore, WO 93/07278 and Koziel *et al.* (Biotechnology 11: 194-200 (1993)) describe techniques for the transformation of elite inbred lines of maize by particle bombardment. This technique utilizes immature maize embryos of

1.5-2.5 mm length excised from a maize ear 14-15 days after pollination and a PDS-1000He Biolistics device for bombardment.

Transformation of rice can also be undertaken by direct gene transfer techniques utilizing protoplasts or particle bombardment. Protoplast-mediated transformation has been described for *Japonica*-types and *Indica*-types (Zhang *et al.* Plant Cell Rep 7: 379-384 (1988); Shimamoto *et al.* Nature 338: 274-277 (1989); Datta *et al.* Biotechnology 8: 736-740 (1990)). Both types are also routinely transformable using particle bombardment (Christou *et al.* Biotechnology 9: 957-962 (1991)). Furthermore, WO 93/21335 describes techniques for the transformation of rice via electroporation.

Patent Application EP 0 332 581 describes techniques for the generation, transformation and regeneration of Pooideae protoplasts. These techniques allow the transformation of *Dactylis* and wheat. Furthermore, wheat transformation has been described by Vasil *et al.* (Biotechnology 10: 667-674 (1992)) using particle bombardment into cells of type C long-term regenerable callus, and also by Vasil *et al.* (Biotechnology 11: 1553-1558 (1993)) and Weeks *et al.* (Plant Physiol. 102: 1077-1084 (1993)) using particle bombardment of immature embryos and immature embryo-derived callus. A preferred technique for wheat transformation, however, involves the transformation of wheat by particle bombardment of immature embryos and includes either a high sucrose or a high maltose step prior to gene delivery. Prior to bombardment, any number of embryos (0.75-1 mm in length) are plated onto MS medium with 3% sucrose (Murashiga & Skoog, Physiologia Plantarum 15: 473-497 (1962)) and 3 mg/l 2,4-D for induction of somatic embryos, which is allowed to proceed in the dark. On the chosen day of bombardment, embryos are removed from the induction medium and placed onto the osmoticum (*i.e.* induction medium with sucrose or maltose added at the desired concentration, typically 15%). The embryos are allowed to plasmolyze for 2-3 hours and are then bombarded. Twenty embryos per target plate is typical, although not critical. An appropriate gene-carrying plasmid (such as pCIB3064 or pSG35) is precipitated onto micrometer size gold particles using standard procedures. Each plate of embryos is shot with the DuPont Biolistics® helium device using a burst pressure of ~1000 psi using a standard 80 mesh screen. After bombardment, the embryos are placed back into the dark to recover for about 24 hours (still on osmoticum). After 24 hrs, the embryos are removed from the osmoticum and placed back onto induction medium where they stay for about a month before

regeneration. Approximately one month later the embryo explants with developing embryogenic callus are transferred to regeneration medium (MS + 1 mg/liter NAA, 5 mg/liter GA), further containing the appropriate selection agent (10 mg/l basta in the case of pCIB3064 and 2 mg/l methotrexate in the case of pSOG35). After approximately one month, developed shoots are transferred to larger sterile containers known as "GA7s" which contain half-strength MS, 2% sucrose, and the same concentration of selection agent.

Tranformation of monocotyledons using *Agrobacterium* has also been described. *See,* WO 94/00977 and U.S. Patent No. 5,591,616, both of which are incorporated herein by reference. *See also,* Negrotto *et al., Plant Cell Reports* 19: 798-803 (2000), incorporated herein by reference.

3. Transformation of Plastids

Seeds of *Nicotiana tabacum* c.v. 'Xanthi nc' are germinated seven per plate in a 1" circular array on T agar medium and bombarded 12-14 days after sowing with 1 μm tungsten particles (M10, Biorad, Hercules, CA) coated with DNA from plasmids pPH143 and pPH145 essentially as described (Svab, Z. and Maliga, P. (1993) *PNAS* 90, 913–917). Bombarded seedlings are incubated on T medium for two days after which leaves are excised and placed abaxial side up in bright light (350-500 μmol photons/m^2/s) on plates of RMOP medium (Svab, Z., Hajdukiewicz, P. and Maliga, P. (1990) *PNAS* 87, 8526–8530) containing 500 μg/ml spectinomycin dihydrochloride (Sigma, St. Louis, MO). Resistant shoots appearing underneath the bleached leaves three to eight weeks after bombardment are subcloned onto the same selective medium, allowed to form callus, and secondary shoots isolated and subcloned. Complete segregation of transformed plastid genome copies (homoplasmicity) in independent subclones is assessed by standard techniques of Southern blotting (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor). BamHI/EcoRI-digested total cellular DNA (Mettler, I. J. (1987) *Plant Mol Biol Reporter* 5, 346–349) is separated on 1% Tris-borate (TBE) agarose gels, transferred to nylon membranes (Amersham) and probed with ^{32}P-labeled random primed DNA sequences corresponding to a 0.7 kb BamHI/HindIII DNA fragment from pC8 containing a portion of the *rps7/12* plastid targeting sequence. Homoplasmic shoots are rooted aseptically on

spectinomycin-containing MS/IBA medium (McBride, K. E. et al. (1994) *PNAS* 91, 7301-7305) and transferred to the greenhouse.

IV. Breeding and Seed Production

A. Breeding

The plants obtained via tranformation with a nucleic acid sequence of the present invention can be any of a wide variety of plant species, including those of monocots and dicots; however, the plants used in the method of the invention are preferably selected from the list of agronomically important target crops set forth *supra*. The expression of a gene of the present invention in combination with other characteristics important for production and quality can be incorporated into plant lines through breeding. Breeding approaches and techniques are known in the art. See, for example, Welsh J. R., *Fundamentals of Plant Genetics and Breeding,* John Wiley & Sons, NY (1981); *Crop Breeding,* Wood D. R. (Ed.) American Society of Agronomy Madison, Wisconsin (1983); Mayo O., *The Theory of Plant Breeding*, Second Edition, Clarendon Press, Oxford (1987); Singh, D.P., *Breeding for Resistance to Diseases and Insect Pests*, Springer-Verlag, NY (1986); and Wricke and Weber, *Quantitative Genetics and Selection Plant Breeding*, Walter de Gruyter and Co., Berlin (1986).

The genetic properties engineered into the transgenic seeds and plants described above are passed on by sexual reproduction or vegetative growth and can thus be maintained and propagated in progeny plants. Generally said maintenance and propagation make use of known agricultural methods developed to fit specific purposes such as tilling, sowing or harvesting. Specialized processes such as hydroponics or greenhouse technologies can also be applied. As the growing crop is vulnerable to attack and damages caused by insects or infections as well as to competition by weed plants, measures are undertaken to control weeds, plant diseases, insects, nematodes, and other adverse conditions to improve yield. These include mechanical measures such a tillage of the soil or removal of weeds and infected plants, as well as the application of agrochemicals such as herbicides, fungicides, gametocides, nematicides, growth regulants, ripening agents and insecticides.

Use of the advantageous genetic properties of the transgenic plants and seeds according to the invention can further be made in plant breeding, which aims at the development of plants with improved properties such as tolerance of pests, herbicides, or stress, improved nutritional value, increased yield, or improved structure causing less loss from lodging or shattering. The various breeding steps are characterized by well-defined human intervention such as selecting the lines to be crossed, directing pollination of the parental lines, or selecting appropriate progeny plants. Depending on the desired properties, different breeding measures are taken. The relevant techniques are well known in the art and include but are not limited to hybridization, inbreeding, backcross breeding, multiline breeding, variety blend, interspecific hybridization, aneuploid techniques, etc. Hybridization techniques also include the sterilization of plants to yield male or female sterile plants by mechanical, chemical, or biochemical means. Cross pollination of a male sterile plant with pollen of a different line assures that the genome of the male sterile but female fertile plant will uniformly obtain properties of both parental lines. Thus, the transgenic seeds and plants according to the invention can be used for the breeding of improved plant lines, that for example, increase the effectiveness of conventional methods such as herbicide or pesticide treatment or allow one to dispense with said methods due to their modified genetic properties. Alternatively new crops with improved stress tolerance can be obtained, which, due to their optimized genetic "equipment", yield harvested product of better quality than products that were not able to tolerate comparable adverse developmental conditions.

B. Seed Production

In seed production, germination quality and uniformity of seeds are essential product characteristics. As it is difficult to keep a crop free from other crop and weed seeds, to control seedborne diseases, and to produce seed with good germination, fairly extensive and well-defined seed production practices have been developed by seed producers, who are experienced in the art of growing, conditioning and marketing of pure seed. Thus, it is common practice for the farmer to buy certified seed meeting specific quality standards instead of using seed harvested from his own crop. Propagation material to be used as seeds is customarily treated with a protectant coating comprising herbicides, insecticides, fungicides, bactericides, nematicides, molluscicides, or mixtures thereof. Customarily used

protectant coatings comprise compounds such as captan, carboxin, thiram (TMTD®), methalaxyl (Apron®), and pirimiphos-methyl (Actellic®). If desired, these compounds are formulated together with further carriers, surfactants or application-promoting adjuvants customarily employed in the art of formulation to provide protection against damage caused by bacterial, fungal or animal pests. The protectant coatings may be applied by impregnating propagation material with a liquid formulation or by coating with a combined wet or dry formulation. Other methods of application are also possible such as treatment directed at the buds or the fruit.

EXAMPLES

The invention will be further described by reference to the following detailed examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Ausubel (ed.), Current Protocols in Molecular Biology, John Wiley and Sons, Inc. (1994); J. Sambrook, *et al.*, Molecular Cloning: *A Laboratory Manual, 3d Ed.,* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press (2001); and by T.J. Silhavy, M.L. Berman, and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984).

Example 1: Cloning of Insect Ecdysone Receptors

PCR primers are designed based on the published sequence for *Manduca sexta* ecdysone receptor (EcR) (genbank accession number U19812 (SEQ ID NOs:1 and 2)) to clone the gene in two halves. RNA is prepared from prepupae larva of *Manduca sexta* using the LiCl/phenol method (Current protocols in molecular biology vol 1, unit 4.3, 1987, John Wiley and sons, publishers) and 1 μg of total RNA is used to prepare cDNA using MMLV reverse transcriptase (Promega). The cDNA is used in a PCR reaction with the primers described above to generate two PCR products for the 5' and 3' halves of the gene. These are subcloned into the pGEM-TA vector (Promega) and sequenced. The two fragments are joined at a unique *Nde*I site within each fragment and ligated into pBS-KS (Stratagene) to create a full length *Manduca sexta* EcR clone named pBSFLMa.

To clone additional lepidopteran ecdysone receptor domains, primers are designed based on homology between insect ecdysone receptors using the Align and Sequencher programs. The primers preferably used are 5'-gtgaagtgaaagcctacgtc-3' (SEQ ID NO:15) (upstream of the ATG) and 5'-tgacgcgctcttcgacatgagac-3' (SEQ ID NO:16) (starting before and including the ATG), and a degenerate primer 5'-ggytgytcrtabccbtcctggta-3' (SEQ ID NO:17) (where y=c/t, r=g/a and b=g/t/c) for the 5' half of each gene and primers 5'-ccbscsathatgcartgtgahc-3' (SEQ ID NO:18) and 5'-ccacrtcccagatctcctcga-3' (SEQ ID NO:19) (where b=g/t/c, h=a/t/c, r=a/g)

for the 3' end of the genes. Total RNA is prepared from prepupae larva from black cutworm (BCW, *Agrotis ipsilon*), European corn borer (ECB, *Ostrinia nubilalis*) and fall army worm (FAW, *Spodoptera frugiperda*) and used for reverse transcriptase and PCR reactions as described above. Products are cloned into pGEM-TA and sequenced. The following partial clones from the ecdysone receptors are obtained: ECB 5' end (SEQ ID NOs:3 and 4) and 3' end (SEQ ID NOs:5 and 6) (collectively comprising A/B, C, D and E domains); FAW 3' end (SEQ ID NOs:7 and 8) (comprising a portion of the D domain and the full E domain); and BCW 3' end (SEQ ID NOs:9 and 10) (comprising a portion of the D domain and the full E domain).

The ecdysone receptors of *Locusta migratoria* and *Chironomus tentans* are cloned using the published genbank sequence AF049136 (SEQ ID NOs:11 and 12), and S60739 (SEQ ID NOs:13 and 14), respectively, to design PCR primers. Partial cDNAs (comprising the C, D and E domains) are isolated, cloned in pGEM-TA and confirmed by sequencing.

Example 2: Construction of VP16 Transactivation Domain Fusions to Ecdysone Receptors (EcR's) and Cloning Into Insect Cell Expression Vectors

A fragment containing the herpes simplex VP16 transactivation domain is cloned from plasmid 35S/USP-VP16 (U.S. Patent No. 5,880,333) using the PCR primers 5'-aagcttgccccccgaccg-3' (SEQ ID NO:20) (placing a *Hind*III site at the 5' end of the domain) and 5'-tctagaggatcctacccaccgtact-3' (SEQ ID NO:21) (placing an inframe stop codon followed by *Bam*HI and *Xba*I sites at the 3' end of the domain).

A *Hind*III site followed by an inframe stop codon and *Bam*HI site is placed at the 3' end of the E domain (ligand binding domain) of each cloned lepidopteran receptor using the oligonucleotides: 5'-ggatcctaaagcttcgtcgtcgacacttcg-3' (SEQ ID NO:22) (for *Manduca sexta* EcR), 5'-ggatcctaaagcttcccgcgggattccacg-3' (SEQ ID NO:23) (for black cutworm EcR); 5'-ggatcctaaagcttcacgtcccagatctcctc-3' (SEQ ID NO:24) (for fall armyworm EcR); 5'-ggatcctaaagcttcacgtcccagatctcctccag-3' (SEQ ID NO:25) (for European cornborer EcR); 5'-ggatcctaaagctttgggatcacatcccag-3' (SEQ ID NO:26) (for *Locusta migratoria* EcR); 5'-ggatcctaaagctttggcgggatggcatga-3' (SEQ ID NO:27) (for *Drosophila melanogaster* EcR); and

5'-ggatcctaaagcttgacatcgccgacatcccagac-3' (SEQ ID NO:28) (for *Chironomus tentans* EcR) in a PCR reaction.

For EcR-VP16 chimeras, the VP16 domain is fused in frame to the 3' end of the E domain of all the cloned ecdysone receptors using the *Hind*III site 3' to each EcR clone and the *Hind*III site engineered at the 5' end of VP16.

For the *Manduca* EcR clone from pBSFLMa (Example 1), a *Bam*HI site is engineered adjacent to the ATG of EcR using the oligonucleotide 5'-ctgcaggatccagacgccgctggtcaaac-3' (SEQ ID NO:29) in a PCR reaction. The *Drosophila* EcR clone from 35S/EcR (Example 1 of U.S. Patent No. 5,880,333) is modified by placing a *Bam*HI site immediately upstream of the ATG with the oligonuclotide 5' ggcaggatccatgaagcggcgctggtc-3' (SEQ ID NO:30) and a *Bgl*II site placed at the 3' end of the *Drosophila* ecdysone receptor ligand binding (E) domain using the oligonucleotide 5'-cggaagatctcgtgcatggccagcgtg-3' (SEQ ID NO:31) in a PCR reaction.

The plasmid pPacU (Courey AJ and Tjian R (1988) *Cell* 55, 887-898) is used as the starting vector for expression constructs. The *Manduca* EcR clone is ligated into pPacU using the *Bam*HI sites flanking the *Manduca* EcR coding region. This expression cassette is referred to as MaFL The *Drosophila* EcR is ligated into the *Bam*HI site of pPacU using the *Bam*HI and *Bgl*II sites. This expression cassette is referred to as DrosFL.

To create a *Drosophila* EcR-VP16 fusion containing only domains C, D and E fused to VP16, a fragment is taken from plasmid 35S/EcR$^{227\text{-}825}$-C1 (Example 5 of U.S. Patent No. 5,880,333) using the *Bam*HI site just upstream of the C domain and the *Kpn*I site just upstream of the E domain and fusing it with the *Drosophila* E-domain-VP16 fusion *Kpn*I-*Bam*HI fragment from above. This fusion (SEQ ID NO:95) is ligated into pPacU using the *Bam*HI sites to create the construct referred to as DDV.

A truncated *Manduca* EcR containing domains C, D and E is fused to VP16. A *Bam*HI site and inframe ATG is engineered just 5' to the C domain using the degenerate primers 5'-ggatccatgggycgagaagaattrtcaccr-3' (SEQ ID NO:32) and 5'-ccacrtcccagatctcctcga-3' (SEQ ID NO:33). This fragment is then joined using the *Nde* site to the 3' end of *Manduca* EcR, which has an engineered *Hind*III site at the 3' end as described above. The reconstructed *Manduca* C, D and E domains are then fused inframe to VP16 with the *Hind*III site and the entire fusion (SEQ ID NO:93) is ligated into pPacU at the *Bam*HI site to create the construct referred to as MMV. Similarly, a *Bam*HI site and inframe ATG is engineered just 5' to the C domain of

European corn borer EcR using the degenerate primers 5'-ggatccatgggycgagaagaattrtcaccr-3' (SEQ ID NO:34) and 5'-ggytgytcrtabccbtcctggta-3' (SEQ ID NO:35). A *Bam*HI site is also engineered at the 5' end of the C domain for *Locusta* EcR using the primers 5'-ggatccatgggccgggaggacctctcgccg-3' (SEQ ID NO:36) and 5'-ggatcctaaagctttgggatcacatcccag-3' (SEQ ID NO:37).

Example 3: Construction Of Reporter Plasmids

A minimal promoter vector is made by ligating a synthetic TATA box sequence oligonucleotide pair, 5'-agcttgagggtataatg-3' (SEQ ID NO:38) and 3'-actcccatattactcga-5' (SEQ ID NO:39), into the *Hind*III site of vector pGL3-basic (Promega) so that the *Hind*III site is recreated 5' to the inserted oligonucleotide and destroyed between the oligonucleotide and the downstream luciferase gene. This vector is designated TATA5.

The binding site from the hsp27 gene (Koelle *et al., Cell* 67(1): 59-77 (1991)) is made with the oligonucleotide pair, 5'-gatccgagacaagggttcaatgcacttgtccaatga-3' (SEQ ID NO:40) and 3'-gctctgttcccaagttacgtgaacaggttactctag-5' (SEQ ID NO:41). This site is multimerized and ligated into the *Bgl*II site of vector TATA-5. One isolate, pCGS154, contains the sequence below in the inserted region, having 2 pairs of sites in inverted orientations. One site has a deletion of a single base from the consensus sequence. The sequence of the inserted region in pCGS154 is shown below:

1 gatccgagac aagggttcaa tgcacttgtc caatgagatc
41 cgagacaagg gttcaatgca cttgtccaat gagatctcat
81 tggacaagtg cattgaacct tgtctcggat ctcattggac
121 aagtgcattg aacccttgtc tcggatc (SEQ ID NO:42).

Example 4: Comparison of *Manduca* and *Drosophila* EcR Activities

An *in vivo* cell based assay is designed to measure transcriptional activation by the receptors of a reporter plasmid. S2 *Drosophila* cells (ATCC CRL-1963) are transiently transfected with luciferase reporter and receptor expression plasmids using the calcium phosphate precipitation procedure (Di Nocera and David (1983) *PNAS* 80, 7095-7098). S2 cells

are plated in 96 well format at a density of 2 x 10^5 in 166.6 μl of Schneider's *Drosophila* media supplemented with antibiotics and 10% heat inactivated fetal bovine serum (GIBO-BRL). The following day, 33.4 μl of a calcium phosphate precipitate containing 3-6 ng of pCGS154 reporter plasmid, 3-6 ng of EcR receptor plasmid along with salmon sperm DNA to a total of 400ng DNA per well is added. Chemical ligands are added 16-24 hours after DNA addition to the cells. Cells are then harvested and extracted 24 hours after chemistry addition following the procedures for the luciferase assay by centrifuging and resuspending the cell pellets in 100μl of cell culture lysis reagent (Promega). Luciferase activity is quantitated using chemiluminescence (Promega) using an analytical luminescence model 2001 luminometer.

S2 cells contain *Drosophila* EcR and USP. The endogenous USP can be used as the heterodimerization partner for EcR or exogenous USP expression plasmid can be added to the assay.

To compare the activities of *Manduca* and *Drosophila* EcR, S2 cells are transiently transfected using the above procedure with the reporter plasmid pCGS154 and either full-length *Drosophila* EcR, DDV, full-length *Manduca* EcR, or MMV. Tebufenozide at 0.2μM and 2μM is used as the chemical ligand. Luciferase assays are performed as described above. All of the results are normalized as a ratio of activity to the light unit value for the pCGS154 reporter without chemistry.

Reporter	EcR vector	No Chemistry	0.2 μM tebufenozide	2 μM tebufenozide
CGS154	DrosFL	1	1	16
CGS154	DDV	1	3	253
CGS154	MaFL	1	100	198
CGS154	MMV	1	1625	1424
CGS154	none	1	1	17

These results demonstrate that the *Drosophila* and *Manduca* receptors have different responses to tebufenozide. The *Manduca* EcR is capable of activating the reporter construct at lower levels of compound (0.2 μM) than is the *Drosophila* EcR. Additionally, the truncated *Manduca* receptor fused to VP16 (MMV) exhibits higher activity than the full-length *Manduca* receptor and the similar truncated *Drosophila* EcR fused to VP16 (DDV).

Example 5: Construction of Chimeric EcR Expression Vectors

The existence of a conserved *Kpn*I site in the *Drosophila* and lepidopteran EcR's just 5' to the E domain allows the domains to be exchanged between the different receptors. For the *Locusta migratoria* and *Chironomus tentans* EcR's, a *Kpn*I site is created in an equivalent position by using the oligonucleotides 5'-ggatccatgaaacttgatgatggcaatatg-3' (SEQ ID NO:43), 5'-tggtaccagataagcttataaataacg-3' (SEQ ID NO:44), 5'-tggtaccaagacggttatgaacagccg-3' (SEQ ID NO:45) and 5'-ggatcctaaagcttgacatcgccgacatcccagac-3' (SEQ ID NO:46) for *Chironomus* and 5'-ggatccatgggccgggaggacctctcgccg-3' (SEQ ID NO:47), 5'-ggatccacacaagcctatgtataag-3' (SEQ ID NO:48), 5'-tgtggtaccagaatgaatatgagtctc-3' (SEQ ID NO:49) and 5'-ggatcctaaagctttgggatcacatcccag-3' (SEQ ID NO:50) for *Locusta* in PCR reactions.

Expression constructs are created in pPacU, containing the C and D domains of one species' EcR fused to the E domain-VP16 fusion from a different species' EcR. These constructs are generated using a *Bam*HI-*Kpn*I fragment from the EcR clones (C and D domain) and a *Kpn*I-*Bam*HI fragment from the EcR-VP16 fusions (E domain-VP16).

EcR chimera	C+D domains	E domain	Activation domain	SEQ ID NO:
MDV	*Manduca sexta*	*Drosophila melanogaster*	VP16	63-64
MBV	*Manduca sexta*	Black cutworm (*Agrotis ipsilon*)	VP16	65-66
MEV	*Manduca sexta*	European corn borer (*Ostrinia nubilalis*)	VP16	67-68
MFV	*Manduca sexta*	Fall armyworm (*Spodoptera frugiperda*)	VP16	69-70
DMV	*Drosophila melanogaster*	*Manduca sexta*	VP16	71-72
DBV	*Drosophila melanogaster*	Black cutworm (*Agrotis ipsilon*)	VP16	73-74
EEV	European corn borer (*Ostrinia nubilalis*)	European corn borer (*Ostrinia nubilalis*)	VP16	75-76
EBV	European corn borer (*Ostrinia nubilalis*)	Black cutworm (*Agrotis ipsilon*)	VP16	77-78
EMV	European corn borer (*Ostrinia nubilalis*)	*Manduca sexta*	VP16	79-80

LLV	*Locusta migratoria*	*Locusta migratoria*	VP16	81-82
LMV	*Locusta migratoria*	*Manduca sexta*	VP16	83-84
MLV	*Manduca sexta*	*Locusta migratoria*	VP16	85-86
CCV	*Chironomus tentans*	*Chironomus tentans*	VP16	87-88
CMV	*Chironomus tentans*	*Manduca sexta*	VP16	89-90
MCV	*Manduca sexta*	*Chironomus tentans*	VP16	91-92
MMV	*Manduca sexta*	*Manduca sexta*	VP16	93-94
DDV	*Drosophila melanogaster*	*Drosophila melanogaster*	VP16	95-96

Example 6: Comparison of EcR Chimera Activities with *Manduca* and *Drosophila* C + D (Hinge + DNA Binding) Domains

The activities of the EcR-VP16 chimeras are compared by transiently transfecting S2 cells as described in Example 3. Tebufenozide is added to the cells at 0.2 μM concentration. Results are expressed as fold activation, a ratio of the activity of the constructs with chemistry added as compared to activity of the constructs without chemistry. All results are normalized to the luciferase activity of the pCGS154 reporter without receptor addition.

Construct	Assay # 1 Fold Activation With Chemistry	Assay # 2 Fold Activation With Chemistry
DDV	1.01	0.68
DMV	8.67	3.72
MDV	1.20	1.18
MMV	87.65	151.06
No receptor	1.0	1.0

Construct	Fold Activation With Chemistry
LLV	0.47
LMV	48.8
No receptor	1.0

These results demonstrate that the *Manduca* EcR E domain confers a higher activity in response to tebufenozide as compared with the E domain of *Drosophila* or *Locusta*. The activity of the *Manduca* E domain is further increased when the C and D domains of *Manduca* EcR are added.

Example 7: Comparison of EcR Chimera Activities with Different E (Ligand Binding) Domains

The activities of the EcR-VP16 chimeras are compared by transiently transfecting S2 cells as in Example 3. Tebufenozide is added to the cells at 0.2 μM concentration. Results are expressed as fold activation of constructs with chemistry as compared to without chemistry. All results are normalized to the luciferase activity of the pCGS154 reporter without receptor.

Construct	Fold Activation With Chemistry Assay #1	Fold Activation With Chemistry Assay #2
No receptor	1.0	1.0
MDV	1.1	1.1
MLV	1.7	1.0
MMV	719.2	595.1
MBV	215.7	211.1
MEV	363.7	131.5
MFV	159.9	175.4

These results demonstrate that constructs containing the E domains of lepidopteran EcR's have a higher response to tebufenozide as compared to E domains from other insect EcR's such as *Drosophila* and *Locusta*.

Example 8: Comparison Of EcR Chimeras With Lepidopteran C, D and E Domains

The activities of the EcR-VP16 chimeras are compared by transiently transfecting S2 cells as in Example 3. Tebufenozide is added to the cells at 0.2 μM concentration. Results are expressed as fold activation of constructs with chemistry as compared to without chemistry. All results are normalized to the luciferase activity of the pCGS154 reporter without receptor.

Construct	Fold Activation With Chemistry Assay #1	Fold Activation With Chemistry Assay #2
No receptor	1.0	1.0
DBV	17.37	3.94
EBV	975.0	273.7
MBV	559.1	866.7

These results demonstrate that the addition of the C and D domains from lepidopteran insects increase the response of the receptor to tebufenozide as compared with the chimera containing the C and D domains from *Drosophila*.

Example 9: Increased Activity of Chimeric EcR's

The activities of the EcR-VP16 chimeras are compared by transiently transfecting S2 cells as in Example 3. Tebufenozide is added to the cells at 0.2 μM concentration. Results are expressed as fold activation of constructs with chemistry as compared to without chemistry addition. All results are normalized to the luciferase activity of the pCGS154 reporter without receptor.

Construct	Fold Activation With Chemistry
CCV	2.0
MCV	553.3
No receptor	1.0

This example demonstrates that specific domains from *Manduca* EcR such as the E domain can be fused with other EcR's to create chimeric EcR's with increased response to tebufenozide.

Example 10: Construction of a Monocot-Expressible Target Expression Cassette Comprising the Firefly Luciferase Reporter Gene and Having Response Elements for the GAL4 DNA Binding Domain

A monocot plant expressible reporter construct comprising the firefly luciferase reporter gene having response elements for the GAL4 DNA binding domain is constructed in the following manner. The luciferase gene is removed from pGL3-basic (Promega) using *Hind*III, followed by filling in of the 5' overhang to create a blunt end using Klenow DNA polymerase, and is subsequently cut with *Xba*I. The luciferase fragment is subcloned into pBluescript (Stratagene) at the *Xba*I and *Sma*I sites. This construct is named pBS-luc. The NOS 3'

transcriptional termination region is cloned into pBS-luc and the resulting construct is named pBS-lucNOS.

The minimal bronze 1 (bz1) promoter (Roth *et al., Plant Cell* 3: 317 (1991)) is cloned into pBS-lucNOS in two parts; one is the TATA region and the other is the intron. The TATA region of the bz1 promoter is cloned by ligating together two sets of annealed oligos, bztata1 (5'-agcttcgcacgcgtggtcgcgcggaataaagcggacacgttgcgcccccag-3' (SEQ ID NO:51)) + bztata2 (5'-ttcgctgggggcgcaacgtgtccgctttattccgcgcgaccacgcgtgcga-3' (SEQ ID NO:52)) annealed to bztata3 (5'-cgaagcccgcacgcatcgcattcgcatcgcatcgcaggtcgcatccgacgctagaag-3' (SEQ ID NO:53)) + bztata4 (5'-aattcttctagcgtcggatgcgacctgcgatgcgatgcgaatgcgatgcgtgcgggc-3' (SEQ ID NO:54)). The annealed set bztata1/2 contains a complementary overhang to the annealed set bztata3/4. The final DNA fragment contains HindIII (5') and EcoRI (3') adapter overhangs. This region is ligated to pBS-lucNOS to form pbz1TATALUC.

The bz1 intron region is cloned by PCR and is designed to have *Eco*RI restriction sites on both the 5' and 3' ends using primers bzintron1 (5'-ccgaattccgggaggacgttggcgaccagggt-3' (SEQ ID NO:55)) and bzintron2 (5'-ccgaattcggtgggagatcagtagcccgtcca-3' (SEQ ID NO:56)).

The two annealed bz1 TATA DNA fragments are mixed with the bz1 intron that is obtained by PCR and digested with *Eco*RI, and the 3 DNA fragments are ligated to pBluescript that is digested with *Hind*III and *Eco*RI. The resulting plasmid containing both the bz1 TATA and intron is named pBS-TATA/intron. The bz1 TATA/intron fragment is obtained from pBS-TATA/intron by digesting with *Hind*III and a partial *Eco*RI digest and this fragment is ligated to pBS-lucNOS at the *Hind*III/*Eco*RI site to form pBS-bz1TATA/intronLUC.

The final step in making the reporter is to insert 10 GAL4 DNA binding sites into pBS-bz1TATALUC and pBS-bz1TATA/intronLUC. The 10 GAL4 elements (Guyer et al. (1998) *Genetics* 149:633-9) are inserted into pBS-bz1TATALUC and pBS-bz1TATA/intronLUC at the *Kpn*I/*Xho*I sites. The resulting vectors are named pCGS228 and pCGS206.

Four additional reporter constructs containing the firefly luciferase reporter gene and having response elements for the GAL4 DNA binding domain are created. These constructs differ in the intron that is inserted in place of the bz1 intron, downstream of the bronze1 TATA region of the promoter. Four additional introns are used: the Adh intron number 1

(SEQ ID NO:106), the Sh intron number 1 (SEQ ID NO:107), the maize ubiquitin intron number 1 (SEQ ID NO:108), and the rice actin intron (SEQ ID NO:109).

For the maize ubi intron (SEQ ID NO:108), the intron is amplified from an expression vector containing the maize ubiquitin promoter with a NOS (nopaline synthase) terminator, using taq DNA polymerase and primers ubi5pst (5'-ggcctgcagggcgttccggtccatggttagggc-3' (SEQ ID NO:110)) and ubi3pst (5'-tccctgcagaagtaacaccaaacaaca-3' (SEQ ID NO:111)). The amplified intron is digested with *Pst*I and ligated to pCGS228 that is digested with *Pst*I to form pCGS215.

The Adh intron 1 (SEQ ID NO:106), containing blunt ends on both ends, is ligated to pCGS228 that is digested with *Eco*RI and blunt ended with Klenow DNA polymerase. The resulting vector is named pCGS216.

The Sh intron 1 (SEQ ID NO:107), containing blunt ends on both ends, is ligated to pCGS228 that is digested with *Eco*RI and blunt ended with Klenow DNA polymerase. The resulting vector is named pCGS217.

The rice actin intron (SEQ ID NO:109) is amplified from an expression vector containing the rice actin promoter with a NOS (nopaline synthase) terminator using taq DNA polymerase and PCR primers act5-ecori (5'-ggcgaattcccggtaaccaccccgcccctc-3' (SEQ ID NO:112)) and act3-ecori (5'-cgcgaattctccctgcagcttctacctacaaaa-3' (SEQ ID NO:113)). The amplified intron is digested with *Eco*RI and ligated to pCGS228 thatis digested with *Eco*RI. The resulting plasmid is named pCGS218.

Example 11: Construction of Expression Vector G(M)MV Containing the Yeast GAL4 DNA Binding Domain, the *Manduca* Ecdysone Receptor Hinge (D) and Ligand Binding (E) Domains, and the Herpes Simplex Virus Protein 16 (VP16) Transcription Activation Domain Driven by the Maize Ubiquitin Promoter

An expression vector containing the maize ubiquitin promoter driving chimeric protein "G(M)MV" comprised of the yeast GAL4 DNA binding domain, the *Manduca* hinge (D) and ligand binding (E) domains, and the herpes simplex VP16 transcriptional activation domain is constructed in the following manner. The GAL4 DNA binding domain is amplified by PCR using the following primers and the plasmid pBD-GAL4 Cam (Stratagene) as template:

GAL4BDforward (5'-aggatccgccaccatgaagctactgtcttc-3' (SEQ ID NO:57)) and GAL4BDreverse (5'-aacgcgtcgatacagtcaactgtctttgacc-3' (SEQ ID NO:58)). The resulting PCR fragment is cloned into pT-Adv (Clonetech) by TA cloning and is referred to as pT-Adv-gal4bd.

The hinge and ligand binding domains (D and E domains) of the *Manduca* EcR with a VP16 activation domain at the C-terminus is amplified by PCR using the following primers and MMV (Example 2) as template: MV forward (5'-aacgcgtatgaggcccgagtgcg-3' (SEQ ID NO:59)) and MV reverse (5'-aaatccggaaatacgactcactatagggcgaat-3' (SEQ ID NO:60)). The resulting PCR fragment is cloned into pT-Adv (Clonetech) by TA cloning and is referred to as pT-Adv-MV.

The GAL4 DNA binding domain is isolated from pT-Adv-gal4bd by digesting with *Mlu*I and *Bam*HI. The *Manduca* D and E domains with the VP16 activation domain are isolated from pT-Adv-MV by digesting with *Sac*II, followed by blunt ending with T4 DNA polymerase, and a subsequent digestion with *Mlu*I. An expression vector containing the maize ubiquitin promoter with a NOS (nopaline synthase) terminator is digested with *Sac*I, followed by blunt ending with T4 DNA polymerase and a subsequent *Bam*HI digestion. The GAL4 DNA binding domain, *Manduca* D and E domains with the VP16 activation domain, and vector fragments are ligated together and the resulting plasmid encoding G(M)MV is named pCGS203. The DNA sequence encoding chimeric receptor G(M)MV is shown as nucleotides 2007-3668 of SEQ ID NO:104 and the amino acid sequence of the encoded receptor is shown as SEQ ID NO:105.

Example 12: Transformation of Maize Suspension Culture Cells with Vectors Encoding *Manduca* EcR Polypeptides Controls Expression of Reporter Polypeptides in the Presence of Chemical Ligands

Maize BMS (Black Mexican Sweet) cultured cells are transfected with pCGS206 and pCGS208 by high velocity microprojectile bombardment. In addition, an expression plasmid containing the maize ubiquitin promoter driving the expression of β-glucuronidase (GUS) is added to the transfection to serve as an internal control to normalize against variations among the samples.

Transfections are treated and cell lysates are made essentially as described in U.S. Patent No. 5,880,333. Tebufenozide (Teb) is added to the cells at a final concentration of 10 μM. Both luciferase and GUS assays are performed with 20 μl of cell lysate for each assay using the Promega Luciferase Kit (Promega cat # E1500) and GUS-Light Kit (Tropix) respectively. Relative light units are determined using a Turner Designs TD 20/20 luminometer. The normalized values (using the GUS reporter control) are listed in the following table.

Constructs	Assay #1 Luciferase	Fold Induction	Assay #2 Luciferase	Fold Induction	Assay #3 Luciferase	Fold Induction
pCGS206	2.32	---	---		0.12	1
pCGS206 + Teb	1.49	0.64	0.79	1	---	---
pCGS206 + pCGS208	0.78	---	1.51	---	3.87	---
pCGS206 + pCGS208 + Teb	28.26	36.2	50.11	33.2	150.89	39.0

The "G(M)MV" *Manduca* EcR (ubi/GAL4-MV) is able to achieve an average of 36-fold induction with tebufenozide.

In addition to tebufenozide, methoxytebufenozide ("MethoxyTeb") is also capable of inducing gene expression in maize BMS cells. Both pCGS206 and pCGS208 are transfected as described above and methoxytebufenozide is added in place of tebufenozide at 25 μM and 10 μM.

Constructs	Assay #1 Luciferase	Fold Activation	Assay #2 Luciferase	Fold Activation
pCGS206	0.81	---	---	---
pCGS206 + MethoxyTeb	0.86	1.06	1.27	---
pCGS206 + pCGS208	15.65	---	11.86	---
pCGS206 + pCGS208 + MethoxyTeb	310.89	19.9	345.5	29.1

Thus, methoxytebufenozide activates the *Manduca* "G(M)MV" EcR (ubi/GAL4-MV) on average 25 fold.

The overall expression level of tebufenozide induction on pCGS203 is dependent on the promoter/intron of the reporter in cultured maize cells. Maize BMS cells are transfected and assayed as described above.

Constructs	Luciferase	Fold Induction
pCGS228 + pCGS203	2.6	---
pCGS228 + pCGS203 + 10μM Teb	6.7	2.6
pCGS206 + pCGS203	0.99	---
pCGS206 + pCGS203 + 10μM teb	69.71	70.3
pCGS215 + pCGS203	1.5	---

pCGS215 + pCGS203 + 10μM teb	27.4	18.8
pCGS216 + pCGS203	4.2	---
pCGS216 + pCGS203 + 10μM teb	87.9	20.9
pCGS217 + pCGS203	1.31	---
pCGS217 + pCGS203 + 10μM teb	18.7	14.3
pCGS218 + pCGS203	3.7	---
pCGS218 + pCGS203 + 10μM teb	20.1	5.4

The addition of various introns changes the overall activation level of tebufenozide induction. Addition of the Adh intron 1 increases the expression level to 87.9 RLU, while the addition of the Sh intron 1 has a moderate expression level of 18.7. Therefore, using different introns regulates the expression level of a desired trait produced from the switch system.

Example 13: Construction of a Vector for Expression of Foreign Genes in Dicot Plants

A dicot expression vector containing the *Arabidopsis* ubiquitin promoter and 5' UTR, a multiple cloning site (MCS), and the nopaline synthase 3' transcriptional termination region (NOS) is generated, and named pCGS417. A cassette containing the *Arabidopsis* ubiquitin

promoter and 5' untranslated region (UTR) and the NOS terminator is cloned into pBluescript using *Xho*I and *Not*I. The MCS is created at the *Bam*HI site between the ubiquitin 5' UTR and the NOS terminator by ligating in the following double stranded oligonucleotide, which has the recognition sequences for restriction enzymes *Sma*I, *Sal*I, *Eco*RI, *Bsp*EI, *Hind*III, and *Xba*I:

5'-gatccccgggtcgacgaattctccggaagcttctaga-3' (SEQ ID NO:61)
3'-gggcccagctgcttaagaggccttcgaagatctctag-5' (SEQ ID NO:62).

Example 14: Construction of a Dicot Expressible Receptor Expression Cassette Encoding the DNA Binding Domain from GAL4 and the Ligand Binding Domain from *Manduca* EcR.

The chimeric receptor fusion GAL4-MV, containing the DNA binding domain from GAL4, the ligand binding (E) domain from *Manduca* EcR, and the viral transactivation domain from VP16, is removed from the monocot expression vector pCGS208 and cloned into pCGS417 using the *Bam*HI restriction sites. This construct is named pCGS431. The VP16 activation domain is removed from the chimeric protein by restriction with *Xba*I and *Hind*III. The 5' overhangs generated in by this digest are filled in using the large (Klenow) fragment of DNA polymerase I, and the vector is recircularized by self-ligation. The resulting vector contains an in-frame transcriptional termination codon immediately downstream of the filled-in *Hind*III sequence. This vector, encoding a GAL4 DNA binding domain-*Manduca* EcR E domain-VP16 fusion protein, is named pCGS432.

Example 15: Transformation of Tobacco Cells with the Reporter and Receptor Constructs Produces a Chemically Inducible Plant Cell System

The GAL4-MV expression vector pCGS432 and the GAL4x10-Luciferase vector pCGS206 are simultaneously delivered into BY2 suspension cells (*Nicotania tobacum* L. cv. Bright Yellow 2) by high velocity microprojectile bombardment. A β-glucuronidase (GUS) vector is included as an internal control for transfection efficiency between samples. Transfected cells are incubated overnight in the presence and absence of the appropriate chemical ligand (tebufenozide (Teb), RH5889) in BY2 liquid culture media. After incubation,

the cells are harvested and lysed by mechanical disruption. Cellular debris is removed by centrifugation at 20,800 g at 4°C for 10 minutes. Cell lysates are assayed for luciferase expression levels using the Promega Luciferase Kit (Promega cat # E1500) using a Turner Designs TD 20/20 luminometer. GUS expression levels are determined using the GUS-Light kit (Tropix). GUS activity is used to normalize luciferase levels to compensate for differences in DNA delivery to each sample.

Receptors	Assay #1		Assay #2		Assay #3	
	Luciferase	Fold Induction	Luciferase	Fold Induction	Luciferase	Fold Induction
None	0.853					
None + Teb.	0.808	0.95				
pCGS432	1.980		0.297		3.395	
pCGS432 + Teb.	11.93	6.0	4.269	14.4	20.49	6.0

These results demonstrate that the luciferase reporter is activated only in the presence of both the GAL4-MV construct and the ligand tebufenozide. Tebufenozide does not activate the reporter in the absence of the EcR receptor.

Example 16: Construction of Dicot Expressible Receptor Expression Cassettes Encoding the DNA Binding Domain from GAL4, the Ligand Binding Domain from *Manduca* EcR, and the C1 Transcriptional Activation Domain

Chimeric receptor fusion proteins are constructed containing the GAL4 DNA binding domain (DBD) and *Manduca* EcR (MecR) ligand binding (E) domain, fused to the C1 transcriptional activation domain in either an N-terminal configuration (C1-GAL4-MEcR), an internal configuration (GAL4-C1-MEcR), or a C-terminal configuration (GAL4-MEcR-C1). The GAL4-MEcR-C1 receptor is designed using the dicot GAL4-MEcR-VP16 expression vector, pCGS431 (Example 14). pCGS431 is digested with *Xba*I and *Hin*dIII to remove the VP16 activation domain. The C1 activation domain is PCR amplified using the primers 5'-ggcaagcttcccaaggccgtgcgg-3' (SEQ ID NO:97), and 5'-ggctctagactacgcaagctgcccggc-3' (SEQ ID NO:98), which include an in-frame *Hin*dIII site at the 5' end of C1 and a translational stop codon and *Xba*I site at the 3' end of C1. The PCR product is digested with both *Hin*dIII and

*Xba*I and cloned into the digested pCGS431. This dicot expression vector encoding the fusion receptor GAL4-MEcR-C1 is named pCGS443.

The GAL4-C1-MEcR chimeric receptor is designed using the GAL4-MEcR expression vector, pCGS432 (Example 14). pCGS432 is digested with *Mlu*I. The C1 activation domain is PCR amplified using primers that include in-frame *Mlu*I sites on both the 5' and 3' ends of C1, 5'-ggcacgcgtcccaaggccgtgcgg-3' (SEQ ID NO:99) and 5'-gccacgcgtcgcaagctgcccggc-3' (SEQ ID NO:100). The PCR product is digested with *Mlu*I and cloned between the GAL4 DBD and the *Manduca* EcR E domain in the *Mlu*I digested pCGS432. This dicot expression vector encoding the fusion receptor GAL4-C1-MEcR is named pCGS442.

The C1-GAL4-MEcR chimeric receptor is also designed using the GAL4-MEcR expression vector, pCGS432. pCGS432 is digested with *Bam*HI, which cuts at the junction between the GAL4 DBD and the *Manduca* EcR E domain. The C1 activation domain is PCR amplified using primers that include in frame *Bam*HI sites on both the 5' and 3' ends of C1, 5'-ccgggatccgccaccatgcccaaggccgtgcgg-3' (SEQ ID NO:101), and 5'-ccgggatcccgcaagctgcccggc-3' (SEQ ID NO:102). The PCR product is digested with *Bam*HI and cloned into the digested pCGS432. This dicot expression vector encoding the fusion receptor C1-GAL4-MEcR is named pCGS441.

Example 17: Transformation of Tobacco Cells with reporter and GAL4-MEcR-C1 Receptor Constructs Produces a Chemically Inducible Plant Cell System

The GAL4-Manduca EcR-C1 expression vectors (pCGS441, pCGS442, and pCGS443) and the GAL4x10-Luciferase vector pCGS206 (Example 10) are simultaneously delivered into BY2 suspension cells (*Nicotiana tobaccum* L. cv. Bright Yellow 2) by high velocity microprojectile bombardment. A β-glucuronidase (GUS) vector is included as an internal control for transfection efficiency between samples. Transfected cells are incubated overnight in the presence and absence of the appropriate chemical ligand (tebufenozide (teb), RH5992) in BY2 liquid culture media. After incubation, the cells are harvested and lysed by mechanical disruption. Cellular debris is removed by centrifugation at 20,800 g at 4°C for 10 minutes. Cell lysates are assayed for luciferase expression levels using the Promega

Luciferase Kit (cat # E1500) using a Turner Designs TD 20/20 luminometer. β-glucuronidase expression levels are determined using the GUS-Light kit (Tropix). GUS activity is used to normalize luciferase levels to compensate for differences in DNA delivery to each sample.

	Assay #1		Assay #2	
Receptors	Luciferase	Fold Induction	Luciferase	Fold Induction
None			3.113	
None + Teb			3.394	1.1
pCGS441	3.548		3.752	
pCGS441 + Teb	68.05	19.2	28.77	7.7
pCGS442	9.287		4.572	
pCGS442 + Teb	50.24	5.4	19.1	4.2
pCGS443	10.84		7.233	
pCGS443 + Teb	161.6	14.9	80.43	11.1

These results demonstrate that all three chimeric receptors (pCGS441, pCGS442, and pCGS443) are able to specifically activate the luciferase reporter upon treatment with the ligand tebufenozide. The GAL4-MEcR-C1 (pCGS443) construct is able to direct a higher level of reporter expression upon ligand induction than are the GAL4-C1-MEcR (pCGS442) or the C1-GAL4-MEcR (pCGS441) chimeric receptors.

Example 18: Construction of a Monocot Reporter Construct for use in *Agrobacterium* Transformation.

A vector for use in *Agrobacterium* transformation of maize containing 10 GAL4 DNA binding elements upstream of the bronze1 TATA containing minimal promoter fused with a fragment of the bronze1 first intron sequence driving luciferase expression is constructed in the following manner. The reporter fragment is excised from pCGS206 (Example 10) using *Kpn*I and *Bgl*II. A modified pBluescript vector containing a BglII site is digested with *Kpn*I and *Bgl*II and the reporter fragment is ligated into modified pBluescript. Using *Kpn*I and *Sma*I, the reporter fragment (SEQ ID NO:103) is then removed from the modified pBluescript vector and directionally cloned into an *Agrobacterium* monocot transformation vector that contains the PMI gene under control of the maize ubiquitin promoter. This plasmid is named pCGS601.

Example 19: Construction of a Monocot Expression Vector Containing the Yeast GAL4 DNA Binding Domain, the *Manduca* EcR Ligand Binding Domain, and the VP16 Transcription Activation Domain for use in *Agrobacterium* Transformation

A vector for use in *Agrobacterium* transformation of maize containing the maize ubiquitin promoter driving a chimeric protein comprised of the GAL4 DNA binding domain, the *Manduca* ligand binding (E) domain, and the herpes simplex viral protein 16 transcriptional activation domain is constructed in the following manner. The GAL4-*Manduca* EcR-VP16 chimeric receptor is excised from plasmid pCGS208 using *Asc*I and *Sfo*I. A plasmid containing a herbicide tolerant *Arabidopsis* protox gene encoding a protox enzyme having a Tyr to Met mutation at AA 426 and a Ser to Leu mutation at AA 305 (sub-sequences 7 and 13 in Table 1B of U.S. Patent No. 6,084,155) downstream of the maize ubiquitin promoter is digested with *Hind*III followed by blunt ending using Klenow DNA polymerase. The plasmid is subsequently digested with *Asc*I and the GAL4- *Manduca* EcR-VP16 DNA fragment (SEQ ID NO:104) is ligated into the plasmid to form pCGS202. Both the ubiquitin-protox cassette and the ubiquitin-GAL4-EcR-VP16 chimera are between the left and right border fragments for *Agrobacterium* transformation.

Example 20: *Agrobacterium*-Mediated Transformation of Maize

Transformation of immature maize embryos is performed essentially as described in Negrotto *et al., Plant Cell Reports* 19: 798-803 (2000). For this example, all media constituents are as described in Negrotto *et al.*, 2000, *supra*. However, various media constituents described in the literature may be substituted.

A. Transformation Plasmids and Selectable Marker

The genes used for transformation are cloned into a vector suitable for maize transformation. Vectors used contain either the phosphomannose isomerase (PMI) gene (Negrotto *et al.*, 2000) or a herbicide-tolerant protoporphyrin oxidase (protox) gene (e.g. that encoding a protox enzyme having a Tyr to Met mutation at AA 426 and a Ser to Leu mutation

at AA 305 (sub-sequences 7 and 13 in Table 1B of U.S. Patent No. 6,084,155)), which allows for selection of transgenic cells with either mannose or herbicide supplemented media respectively. In the case of single genes - e.g. a reporter construct or an induction system – one strain of *Agrobacterium* is utilized in an experiment. For transfer of both genes, they are either cloned into a single T-region on one plasmid, or they are cloned onto separate plasmids and the two *Agrobacterium* strains harboring these separate plasmids are mixed 1:1 before inoculation followed by selection for both marker genes. Alternatively, the genes are cloned separately onto plasmids with compatible origins of replication and transformed into a single *Agrobacterium* strain that is used for transformation, or transformed plants from single transgenes are crossed to produce progeny with both traits.

B. Preparation of *Agrobacterium tumefaciens*

Agrobacterium strain LBA4404 (pSB1) containing the plant transformation plasmid is grown on YEP (yeast extract (5 g/L), peptone (10g/L), NaCl (5g/L),15g/l agar, pH 6.8) solid medium for 2 – 4 days at 28°C. Approximately 0.8X 10^9 *Agrobacteria* are suspended in LS-inf media supplemented with 100 μM As (Negrotto, *et al.*, 2000). Bacteria are pre-induced in this medium for 30-60 minutes.

C. Inoculation

Immature embryos from A188 or other suitable maize genotype are excised from 8 – 12 day old ears into liquid LS-inf + 100 μM As. Embryos are rinsed once with fresh infection medium. *Agrobacterium* solution is then added and embryos are vortexed for 30 seconds and allowed to settle with the bacteria for 5 minutes. The embryos are then transferred scutellum side up to LSAs medium and cultured in the dark for two to three days. Subsequently, between 20 and 25 embryos per petri plate are transferred to LSDc medium supplemented with cefotaxime (250 mg/l) and silver nitrate (1.6 mg/l) and cultured in the dark at 28°C for 10 days.

D. Selection of Transformed Cells and Regeneration of Transformed Plants

PMI selection: Immature embryos, producing embryogenic callus, are transferred to LSD1M0.5S medium. The cultures are selected on this medium for 6 weeks with a subculture

step at 3 weeks. Surviving calli are transferred either to LSD1M0.5S medium to be bulked-up or to Reg1 medium supplemented with mannose. Following culturing in the light (16 hour light/ 8 hour dark regimen), green tissues are then transferred to Reg2 medium without growth regulators and incubated for 1-2 weeks. Plantlets are transferred to Magenta GA-7 boxes (Magenta Corp, Chicago Ill.) containing Reg3 medium and grown in the light. After 2-3 weeks, plants are tested for the presence of the PMI genes and other genes of interest by PCR. Positive plants from the PCR assay are transferred to the greenhouse.

Herbicide selection: Selection conditions are essentially performed as described for PMI selection and regeneration with the following media modifications. Silver nitrate is used in both initiation and selection media and sucrose is used at 30 g/L. A protox inhibitory herbicide (U.S. Patent No. 6,084,155) is added to the media at 5nM for initiation and primary selection, 500nM for second selection and 750nM for the final selection. Regeneration 1 is carried out on media supplemented with 50nM herbicide with no herbicide selection in subsequent regeneration media.

Combined selection: When mixed infections are used, selection and regeneration are accomplished with both mannose and herbicide containing media.

Example 21: Chemical Induction of Transgenic Maize Plants Containing both the GAL4-*Manduca* EcR Chimeric Protein and a Luciferase Reporter

Transgenic maize plants containing pCGS601 and pCGS202 from Examples 18 and 19, respectively, are chemically induced with tebufenozide (teb, RH5992). A formulation mixture containing 10% of a 2.0 mM tebufenozide solution in ethanol and 1% surfactant is applied directly to the leaves of the transgenic maize plants. The solution is left on the leaves between 18 and 40 hours. Leaves are then frozen in liquid nitrogen and ground to a powder. Subsequently, leaves are homogenized in cell culture lysis reagent (CCLR; 25 mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM 1,2-diaminoclyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, and 1% triton X-100). After spinning for 7 minutes at 14,000g to pellet the cell debris, 20 µl of the supernatant is assayed for luciferase activity by the standard method (as described in the preceding examples).

Six plants are treated with chemistry: three with ethanol as control and three with tebufenozide. After 40 hours, plants are assayed for luciferase activity.

Plant Number	Treatment	Activity
1	Ethanol	280.05
2	Ethanol	6.149
3	Ethanol	119.4
4	Tebufenozide	2729
5	Tebufenozide	745.3
6	Tebufenozide	2373

The transgenic maize plants containing the luciferase reporter and the GAL4-*Manduca* EcR-VP16 chimeric protein demonstrate a significant induction with tebufenozide above the ethanol controls.

To determine fold induction with tebufenozide, leaves of individual plants are compared for activity. Using the same plant, one leaf is treated with ethanol and another with tebufenozide. The plants are incubated for 18 hours and assayed for luciferase activity as described above.

Plant Number	Treatment	Activity	Fold Induction
1	Tebufenozide	118.1	11
2	Ethanol	29.35	
2	Tebufenozide	180	6
3	Ethanol	9.98	
3	Tebufenozide	369.1	37

These results demonstrate approximately 10 to 40 fold induction after treatment with tebufenozide.

Example 22: Chemical Induction by Tebufenozide on Various Tissues of Transgenic Plants Containing pCGS202 and pCGS601

Transgenic plants harboring DNA from the plasmids pCGS202 and pCGS601 are chemically induced with tebufenozide as in Example 21, using 2.0 mM RH5992 formulated in 1% surfactant. This mixture is applied directly to one leaf of a plant while a control

formulation mixture containing ethanol instead of tebufenozide is applied to another leaf. Treatments are left on the leaves between 24-48 hours. Plants are then treated by spraying the leaves with a mixture containing 7.5 mM luciferin and 1% surfactant and incubating for 5 minutes. Either whole plants or tissues are placed into a dark box and photon emission is monitored using a digital camera and software that counts the accumulated photons that are emitted. Both the image of the tissue and the total photon count is recorded.

Plants at V5 stage are treated as above. The plants treated with tebufenozide emit substantial amounts of light compared with those treated with ethanol only. The table below contains actual counts of leaf samples from maize plants transformed with pCGS601 and pCGS202.

Treatment on Leaf	Total Photons Counted
Ethanol	1612
Tebufenozide	187,784
Fold Induction	116 Fold

Late stage post pollinated maize plants are also treated as above. Leaves are analyzed as above and the total photon counts are recorded in the table below.

Treatment on Leaf	Total Photons Counted
Ethanol	3,681
Tebufenozide	110,766
Fold Induction	30 Fold

Similar treatment and results are obtained with the following tissue: roots, tassel, anther, stalk, embryo, and seed.

Example 23: Construction of Expression Vectors Containing the Yeast GAL4 DNA Binding Domain, Combinations of D + E domains (hinge + ligand binding domains), and the Herpes Simplex Virus Protein 16 (VP16) Transcription Activation Domain Driven by the Maize Ubiquitin Promoter

Constructs are cloned by insertion of receptor domains (*Mlu*I, *Pvu*II or *Eco*RV) and the yeast GAL4 DNA Binding Domain (*Bam*HI, *Mlu*I) into an expression vector containing the

maize ubiquitin promoter with a NOS (nopaline synthase) terminator (*Sac*I blunt, *Bam*HI) via three-way ligation. Receptor domains are cloned by PCR amplification of D (hinge), E (ligand binding domain), and VP16 from constructs described in Example 5 (MBV (SEQ ID NOs:65-66); MFV (SEQ ID NOs:69-70); MEV (SEQ ID NO:67-68); EEV (SEQ ID NOs:75-76); and EMV (SEQ ID NOs:79-80)). Forward primers Manduca_Hinge-f (5'-gctcgacgcgtatgaggcccgagtgcgtcgtcccagag-3' (SEQ ID NO:114)) and ECB_Hinge-f (5'-gctcgacgcgtatgaggcccgagtgcgtggtgccag-3' (SEQ ID NO:115)) place a *Mlu*I restriction site at the 5' end of the D domain. Reverse primers VP16-r (*Pvu*II) (5'-tgccagctgctagaggatcctacccaccgtactcg-3' (SEQ ID NO:116)) and VP16-r (*Eco*RV) (5'-tgcgatatcggatcctacccaccgtactcgtcaattcc-3' (SEQ ID NO:117)) place either a *Pvu*II or *Eco*RV site as indicated at the 3' end of the E domain. PCR reactions (50 µl volume) contain 1x buffer, 0.1 µg DNA template, 200 µM dNTPs, 400 nM of both a forward and a reverse primer, and 2.5 U DNA Polymerase. PCR reaction conditions are as follows: 5 minutes at 94°C, 30 cycles of 1 minute at 94°C, 1 minute at 65°C, 1 minute at 72°C, then 10 minutes at 72°C. Amplified cDNA fragments include: 1) (M)BV (1215 bp), consisting of the *Manduca* D domain, the BCW E domain, and VP16; 2) (M)EV (1206 bp), consisting of the *Manduca* D domain, the ECB E domain, and VP16; 3) (M)FV (1211 bp), consisting of the *Manduca* D domain, the FAW E domain, and VP16; 4) (E)EV (1221 bp), consisting of the ECB D domain, the ECB E domain, and VP16; 5) (E)MV (1244 bp), consisting of the ECB D domain, the *Manduca* E domain, and VP16. The yeast GAL4 DNA Binding Domain is obtained by digestion with *Bam*HI and *Mlu*I then isolation of a 453 bp product. An expression vector containing the maize ubiquitin promoter with a NOS (nopaline synthase) terminator (*Sac*I blunt, *Bam*HI) backbone is prepared by digestion with *Sac*I, removal of 3' overhangs with T4 DNA Polymerase, and then digestion with *Bam*HI to yield a 4969 bp product.

EcR chimera	DNA Binding Domain	D domain	E domain	Activation domain	SEQ ID NO:
G(M)MV	GAL4	*Manduca sexta*	*Manduca sexta*	VP16	104-105
G(M)BV	GAL4	*Manduca sexta*	Black cutworm (*Agrotis ipsilon*)	VP16	118-119
G(M)EV	GAL4	*Manduca sexta*	European corn borer (*Ostrinia nubilalis*)	VP16	120-121
G(M)FV	GAL4	*Manduca sexta*	Fall armyworm (*Spodoptera frugiperda*)	VP16	122-123
G(E)EV	GAL4	European corn borer (*Ostrinia nubilalis*)	European corn borer (*Ostrinia nubilalis*)	VP16	124-125
G(E)MV	GAL4	European corn borer (*Ostrinia nubilalis*)	*Manduca sexta*	VP16	126-127

Example 24: Combinations of D + E domains (Hinge + Ligand Binding Domains) Alter the Level of Unliganded Background and Overall Expression Upon Tebufenozide Induction

Maize BMS cells are transfected and assayed as in Example 12.

Experiment 1	Normalized Average (EtOH)	Normalized Average (20 μM Teb)	Fold Induction with Teb Relative to Reporter with EtOH	Fold Induction with Teb Relative to Receptor with EtOH
Reporter	3.671	2.916	1	1
G(M)MV	6.889	305.622	83	44
G(M)BV	8.701	298.862	81	34
G(M)EV	6.965	436.532	119	63
G(M)FV	3.766	192.440	52	51
G(E)EV	7.601	436.430	119	57
G(E)MV	14.499	496.184	135	34
Experiment 2	Normalized Average (EtOH)	Normalized Average (20 μM Teb)	Fold Induction with Teb Relative to Reporter with EtOH	Fold Induction with Teb Relative to Receptor with EtOH
Reporter	.418	.102	1	1
G(M)MV	1.721	25.604	61	15
G(M)BV	1.539	39.677	95	26
G(M)EV	0.028	58.166	139	58
G(M)FV	0.484	23.896	57	24
G(E)EV	2.394	41.446	99	17
G(E)MV	2.537	39.716	95	16

Depending on the combination, D + E domains (hinge + ligand binding domain) affect the level of unliganded background and the level of tebufenozide induction. Such variation provides the opportunity to establish specific conditions of gene expression output by altering receptor domains. The highest fold induction is obtained from EcR chimera G(M)EV having a *Manduca* D (hinge) domain and a European Corn Borer E (ligand binding) domain.

Example 25: Construction of Expression Vectors Containing the Yeast GAL4 DNA Binding Domain, the *Manduca* Hinge (D) and Ligand Binding (E) Domains, and Alternative Activation Domains Derived from Plant Transcription Factors

An expression vector containing the maize ubiquitin promoter with a NOS (nopaline synthase) terminator is used as a vector backbone. The G(M)M (GAL4 DNA Binding Domain fused to the *Manduca* EcR Hinge and Ligand Binding Domain) chimeric receptor nucleotide coding sequence (SEQ ID NOs:128-129) is generated by digesting the dicot expression vector pCGS443 (Example 16) with *Hind*III and *Bam*HI. These reagents are used for cloning-in of additional transcriptional activation domains in frame with the G(M)M chimeric receptor, as detailed below.

The maize C1 activation domain is PCR amplified using the following primers to engineer a *Hind*III site and a *Sac*I site on the 5' and 3' ends respectively of the C1 activation domain: HindIII C1 5' (5'-aaaaaaagcttcccaaggccgtgcggtg-3' (SEQ ID NO:130)) and SacI C1 3' (5'-aaaaagagctcttacgcaagctgcccggcc-3' (SEQ ID NO:131)).

Expression vector pUbi-G(M)MC (pCGS672) is obtained via a three-way ligation between the C1 PCR product digested with *Hind*III and *Sac*I, an expression vector containing the maize ubiquitin promoter with a NOS (nopaline synthase) terminator digested with *Bam*HI and *Sac*I, and the G(M)M *Bam*HI/*Hind*III fragment.

The maize Dof1 transcriptional activation domain is cloned via RT-PCR from total maize RNA using the following primers: HindIII Dof1 5' (5'-aaaaaagcttgagctcgccaccgc-3' (SEQ ID NO:132)) and BamHI Dof1 3' (5'-aaaaaggatcctcacgggaggttgag-3' (SEQ ID NO:133)).

Expression vector pUbi-G(M)MD (pCGS678) is obtained via a three-way ligation between the Dof1 PCR product digested with *Hind*III and *Bam*HI, an expression vector containing the maize ubiquitin promoter with a NOS (nopaline synthase) terminator digested with *Bam*HI, and the G(M)M *Bam*HI/*Hind*III fragment.

EcR chimera	DNA Binding Domain	D domain	E domain	Activation domain	SEQ ID NO:
G(M)MV	GAL4	*Manduca sexta*	*Manduca sexta*	VP16	104-105
G(M)MC	GAL4	*Manduca sexta*	*Manduca sexta*	C1	134-135
G(M)MD	GAL4	*Manduca sexta*	*Manduca sexta*	Dof1	136-137

Example 26: Transformation of Maize Suspension Culture Cells with Vectors Encoding the G(M)MV, G(M)MC, and G(M)MD Chimeric Receptors in Addition to a Luciferase Reporter Gene Vector

Transformation of maize suspension cells, chemical treatment, and reporter activity assays are performed as described in Example 12. Maize BMS (Black Mexican Sweet) cultured cells are transfected with pCGS206 (pBS-bz1TATA/intronLUC), pCGS203 (G(M)MV), pCGS672 (G(M)MC), and pCGS678 (G(M)MD) by high velocity microprojectile bombardment. In addition, an expression plasmid containing the maize ubiquitin promoter driving the expression of β-glucuronidase (GUS) is added to the transfection to serve as an internal control to normalize against variations among the samples.

Transfections are treated and cell lysates are made essentially as described in U.S. Patent No. 5,880,333. Tebufenozide (Teb) is added to the cells at a final concentration of 10 μM. Both luciferase and GUS assays are performed with 20 μl of cell lysate for each assay using the Promega Luciferase Kit (Promega cat # E1500) and GUS-Light Kit (Tropix) respectively. Relative light units are determined using a Turner Designs TD 20/20 luminometer. The normalized values (using the GUS reporter control) are listed in the following table.

Constructs	Experiment #1 Luciferase	Fold Induction	Experiment #2 Luciferase	Fold Induction
Reporter alone	1.00	---	1.00	---
G(M)MV	5.97	---	3.89	---
G(M)MV + Teb	99.49	16.7	136.41	35.1
G(M)MC	2.05	---	1.58	---
G(M)MC + Teb	53.20	26.0	28.05	17.8
G(M)MD	2.14	---	1.18	---
G(M)MD + Teb	6.37	3.0	9.14	7.8

Example 27: Construction of Monocot Receptor Expression Cassettes Encoding the GAL4 DNA Binding Domain, the Hinge and Ligand Binding Domains from *Manduca* EcR, and the VP16 Transcriptional Activation Domain in Various Configurations

Chimeric receptor fusion proteins are constructed containing the GAL4 DNA Binding Domain (DBD) and *Manduca* EcR Hinge (D) and Ligand Binding (E) domains (MEcR), fused to the VP16 transcriptional activation domain in either an N-terminal configuration (VP16-GAL4-MEcR), an internal configuration (GAL4-VP16-MEcR) or a C-terminal configuration (GAL4-MEcR-VP16). The monocot G(M)MV expression construct (GAL4-MEcR-VP16) is described (pCGS203, Example 11).

For construct VG(M)M (VP16-GAL4-MEcR), the following primers are used to generate GAL4-MEcR-Stop: Gal4 DBD 5' (5'-aaaaactagtaagctactgtcttctatcg-3' (SEQ ID NO:138)) and MEcR 3' w/stop (5'-ggatcctaaagcttcgtcgtcgacacttcg-3' (SEQ ID NO:139)). The ATG/Kozak VP16 activation domain cassette is amplified from pCGS203 using the following primers: VP16 5' ATG/Kozak (5'-aaaaaggatccgccaccatgcacgtgaagcttgccccccgac-3' (SEQ ID NO:140)) and VP16 3' no stop (5'-aaaaaactagtcacgtgcccaccgtactcgtcaattcc-3' (SEQ ID NO:141)). The monocot expression vector is generated by performing a three-way ligation between an expression vector containing the maize ubiquitin promoter with a NOS (nopaline synthase) terminator digested with *Bam*HI, Gal4-MEcR-Stop digested with *Spe*I and *Bam*HI,

and ATG/Kozak VP16 digested with *Spe*I and *Bam*HI, resulting in pUbi:VP16-Gal4 DBD-MEcR LBD (VG(M)M; pCGS686). The DNA sequence encoding chimeric receptor VG(M)M is shown as SEQ ID NO:142 and the amino acid sequence of the encoded receptor is shown as SEQ ID NO:143.

For construct GV(M)M (GAL4-VP16-MEcR), the following primer is used in conjuction with primer MEcR 3' w/stop (SEQ ID NO:139) to generate ATG/Kozak Gal4 DBD-MEcR-Stop: Gal4 DBD 5' ATG/Kozak (5'-caaggatccgccaccatgaagctactgtcttctatcg-3' (SEQ ID NO:144)). This Gal4 DBD-MEcR product is TA cloned into pT-Adv, and digested out with *Bam*HI. This is then cloned into an expression vector containing the maize ubiquitin promoter with a NOS (nopaline synthase) terminator that has been cut with *Bam*HI to produce pUbi-Gal4 DBD-MEcR. The VP16 activation domain is generated by PCR amplification from pCGS203 using the following primers: VP16 PmlI 5' (5'-aaaaacacgtgcaagcttgccccccgac-3' (SEQ ID NO:145)) and VP16 PmlI 3' (5'-aaaaacacgtgttcccaccgtactcgtcaattcc-3' (SEQ ID NO:146)). The resulting PCR product is digested with *Pml*I and cloned into pUbi-Gal4 DBD-MEcR that has also been digested with *Pml*I. The resulting GV(M)M construct has the Zm Ubi promoter driving expression of the Gal4 DBD-VP16-MEcR chimeric receptor (pCGS687). The DNA sequence encoding chimeric receptor GV(M)M is shown as SEQ ID NO:147 and the amino acid sequence of the encoded receptor is shown as SEQ ID NO:148.

Example 28: Transformation of Maize Suspension Culture Cells with G(M)MV, GV(M)M, and VG(M)M Receptors Controls Expression of a Reporter in the Presence of a Chemical Ligand

Maize BMS cells are bombarded with reporter vector pCGS206 and chimeric receptors and treated with tebufenozide essentially as described in Example 12. Transfections are treated and cell lysates are made essentially as described in U.S. Patent No. 5,880,333. Tebufenozide (Teb) is added to the cells at a final concentration of 10 μM. Both luciferase and GUS assays are performed with 20 μl of cell lysate for each assay using the Promega Luciferase Kit (Promega cat # E1500) and GUS-Light Kit (Tropix) respectively. Relative

light units (RLU) are determined using a Turner Designs TD 20/20 luminometer. The normalized values (using the GUS reporter control) are listed in the following table.

Receptors	Experiment #1 RLU	Fold Induction	Experiment #2 RLU	Fold Induction
None	1.0	---	1.0	---
None + Teb	1.0	1.0	1.0	1.0
G(M)MV	3.73	---	3.17	---
G(M)MV + Teb	37.56	10.1	66.96	21.1
GV(M)M	1.71	---	1.77	---
GV(M)M + Teb	13.33	7.8	14.15	8.0
VG(M)M	2.21	---	2.52	---
VG(M)M + Teb	58.02	26.3	33.69	13.4

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the present invention.